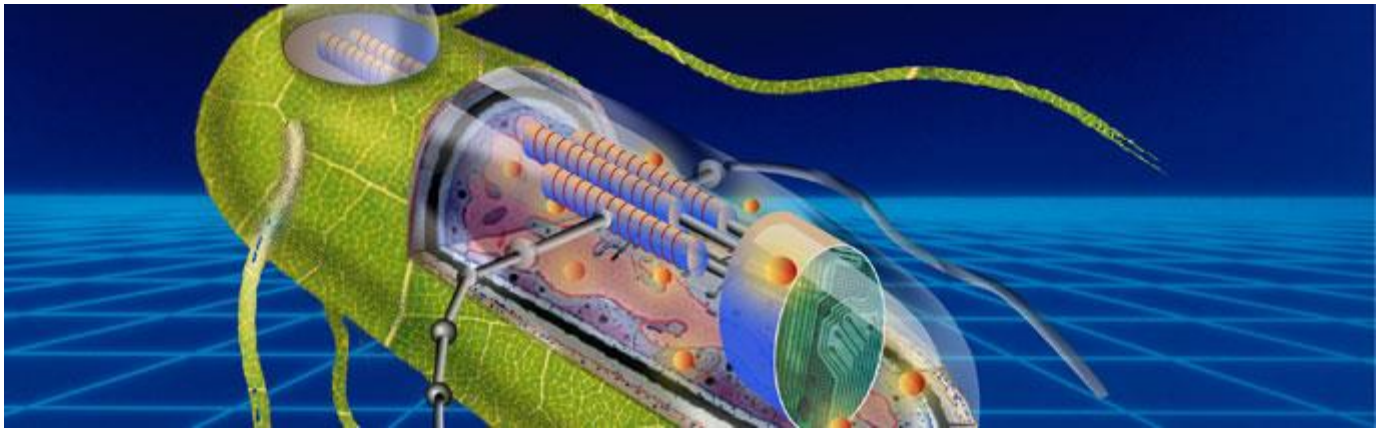
LOSCHMIDT LABORATORIES

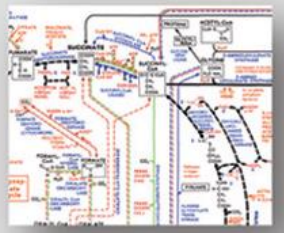
Bi9540 Biotechnology and practical use of algae and fungi

Lecture 13 – Engineering of algae and fungi



Modeling & Design

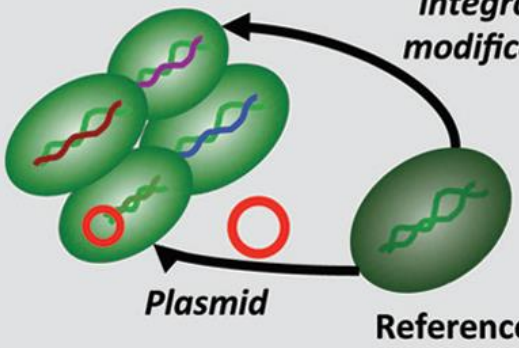
Genome-scale metabolic models
Kinetic models
Thermodynamics
Flux balancing



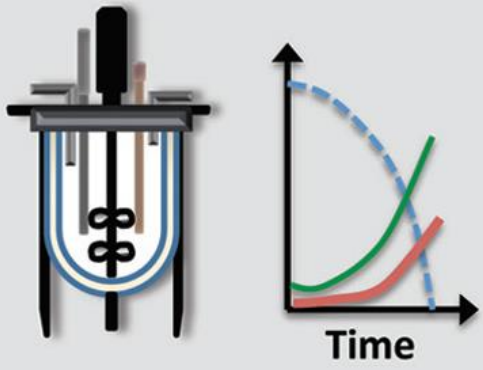
$$\mathbf{Tv = b}$$

$$\frac{dC^{prod}}{dt} = k_f C_{react} - k_r C_{prod}$$

Strain Construction

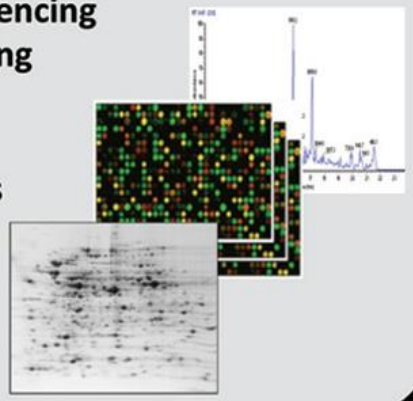


Fermentation

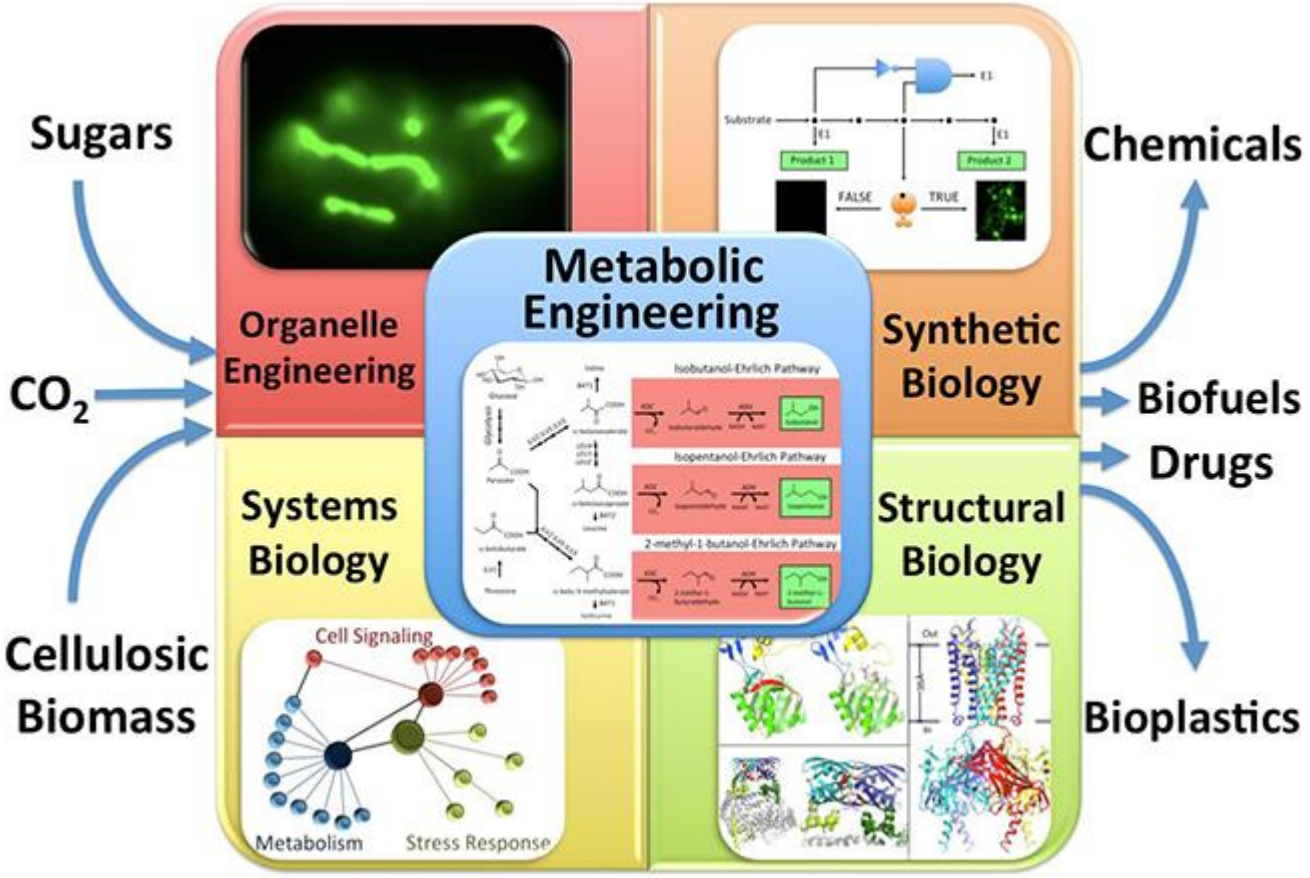


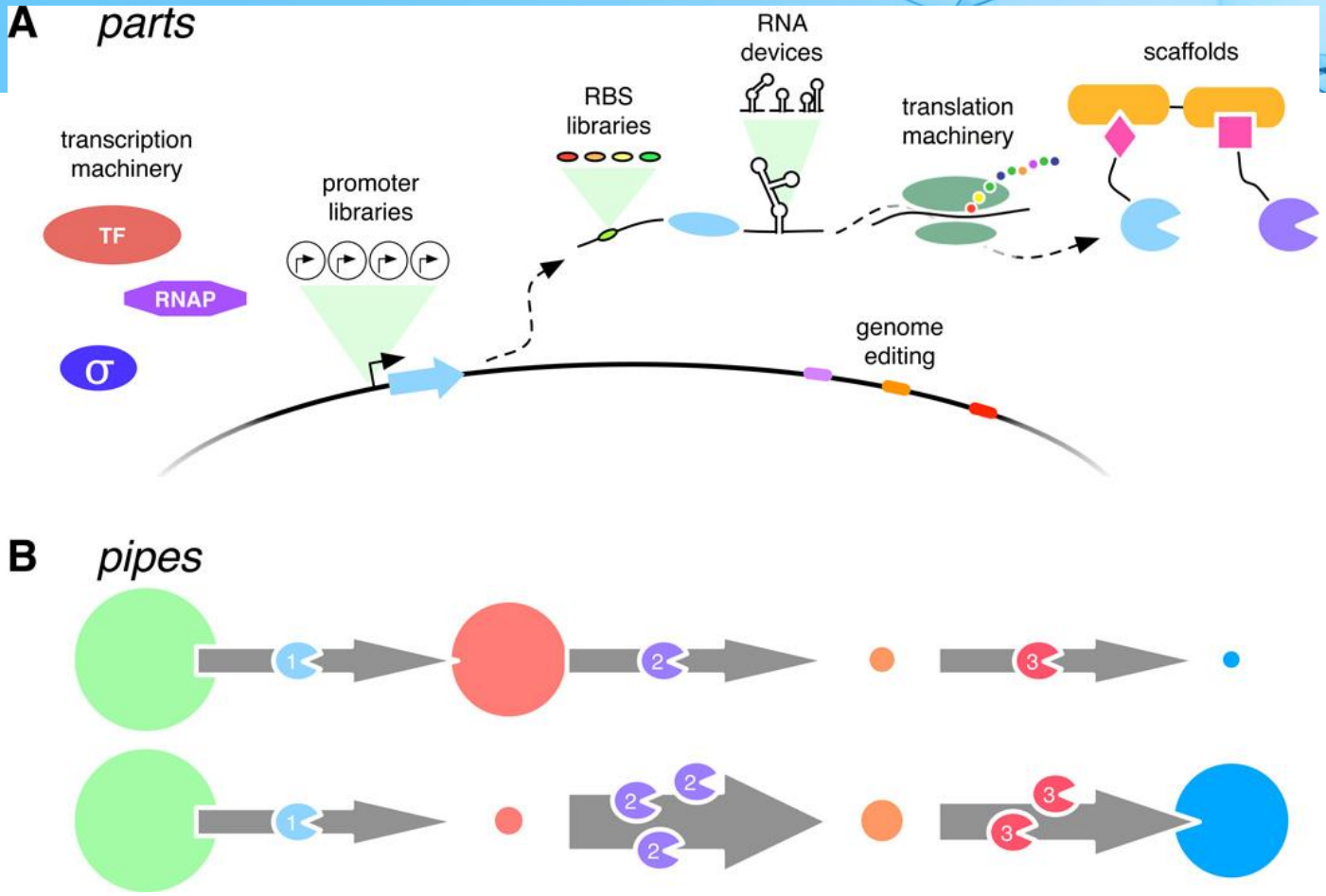
Omics Analysis

Genome sequencing
RNA sequencing
DNA arrays
Proteomics
Metabolomics
Fluxomics



Metabolic engineering and synthetic biology





A) Synthetic biologists use a variety of parts to adjust the functioning of metabolic pathways. Transcription machinery, enzyme promoters, ribosome binding sites (RBS), and translational machinery can be modified to adjust the concentration of an enzyme. RNA devices can modulate mRNA degradation and translation efficiency. Pathway enzymes can be assembled on scaffolds to optimize the spatial organization of a pathway. Genome editing approaches can be used to adjust host metabolism to improve flux through the target pathway. (B) A "pipe" of key pathway enzymes can be tuned to increase product titers. In this conceptual example, enzyme flux is represented by the size of the gray arrows. Metabolite concentrations are represented by the size of the circles between enzymes. In this example, increasing the concentration of the second and third enzymes in the pathway increases the titer of the product. Note that decreasing the concentration of intermediate metabolites can be beneficial; this is often the case when intermediates are harmful to the host cell. Increasing enzymes does not always improve product titers and can in fact be detrimental. In this review, we present synthetic biological parts that enable optimization of metabolic pipes.

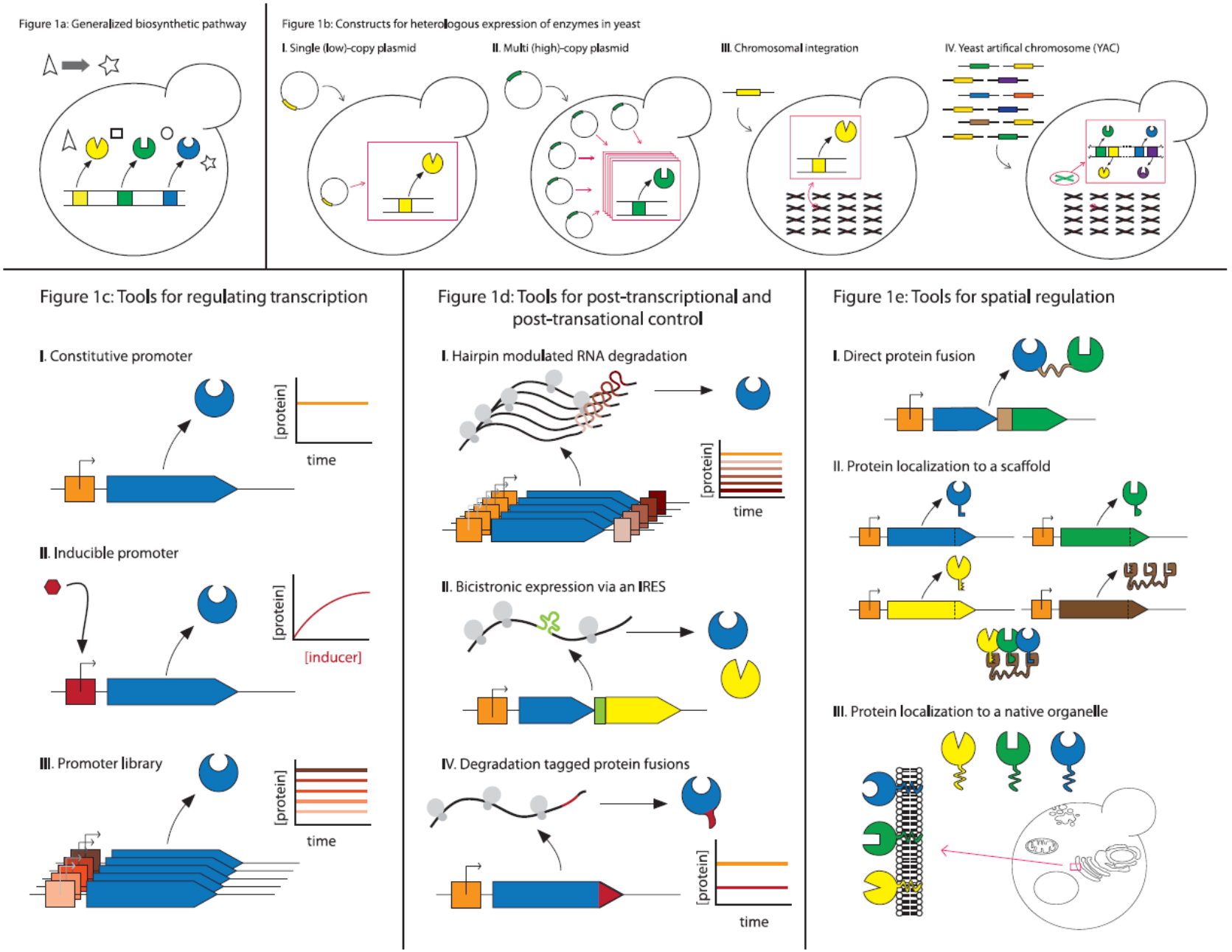


Fig. 1. Tools for controlling enzyme expression in yeast. (a) Metabolic engineering efforts in yeast utilize an array of tools for the expression and regulation of heterologous genes in *Saccharomyces cerevisiae*. Tools enabling heterologous enzyme expression (b), transcriptional regulation (c), post-transcriptional and post-translational regulation (d), and spatial regulation (e) in yeast are illustrated.

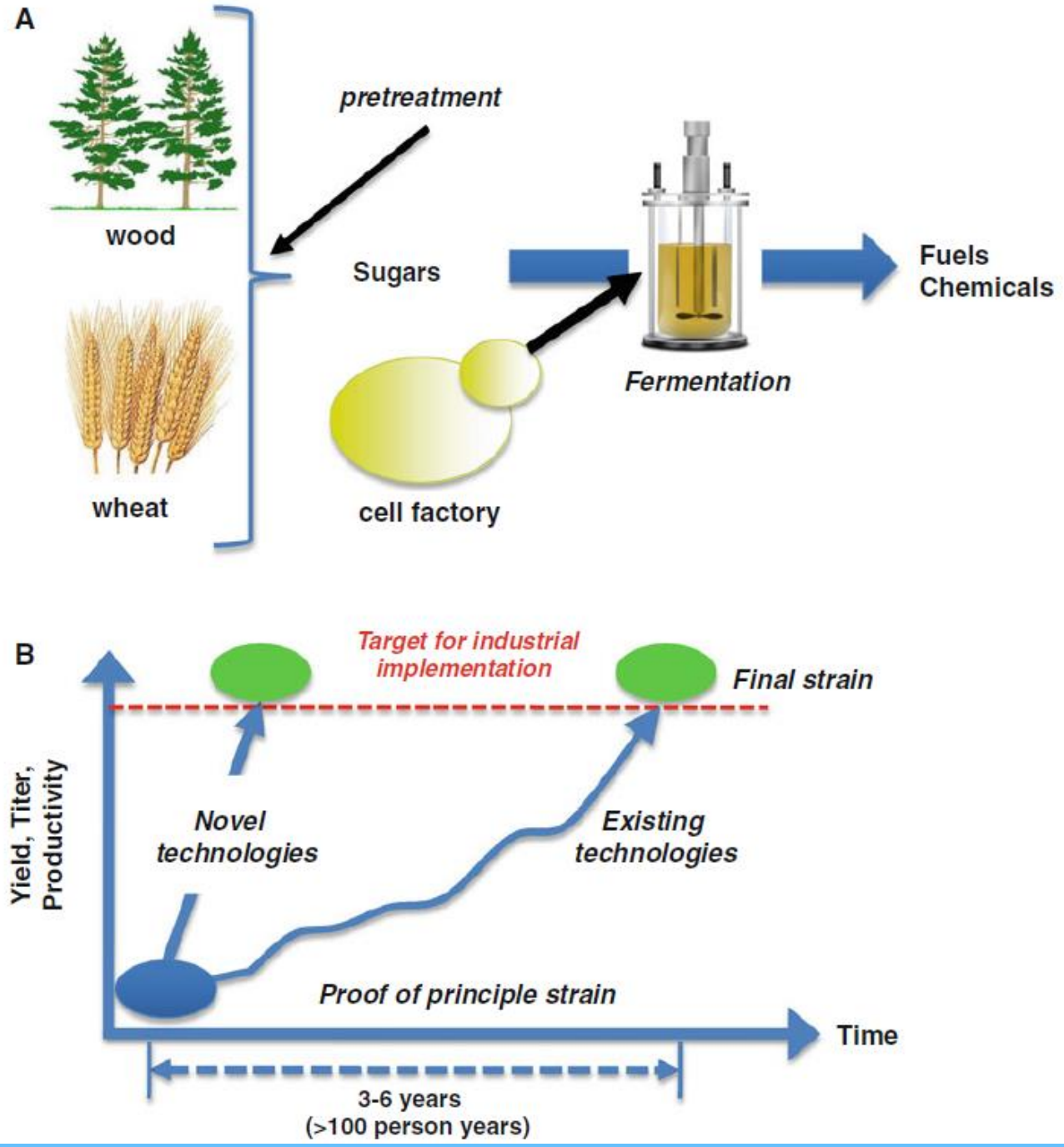


Fig. 1 Illustration of the biorefinery concept and the development time of novel bioprocesses. **a** In a biorefinery, plant-based feed-stocks such as sugarcane, corn, wheat, or biomass are converted into sugars that are subsequently used for microbial fermentations. In the fermentation process, cell factories convert the sugars into fuels and chemicals. **b** The development of cell factories is the central research and development process in connection with the development of a novel bioprocess. Construction of an efficient cell factory requires large investment, in particular in connection with bringing the cell factory from proof-of-principle stage where it is producing small amounts of the desired product to a final strain that produces the product at yields, titers, and productivities that make the process financially competitive with fossil fuel-based processes

Secondary metabolites

- Fungi employ great diversity of metabolic pathways for production of secondary metabolites
- Engineering of the existing pathways is more efficient than creation of novel pathways in other organisms
- Most prominent targets of fungal engineering are:
  - Biofuels
  - Pharmaceutical precursors
  - Fine chemicals

Table 1. Phenolic biosynthetic genes used to build production pathways in yeast

Abbreviation	Gene name	Species	Publications
4CL	4-Coumarate:CoA-ligase	*Petroselinum crispum*, *Nicotiana tabacum*, *Arabidopsis thaliana*, Poplar hybrid (*P. trichocarpa x P. deloides*), *Glycine max*	Becker *et al.* (2003), Jiang *et al.* (2005), Leonard *et al.* (2005), Yan *et al.* (2005), Beekwilder *et al.* (2006), Zhang *et al.* (2006), Yan *et al.* (2007), Trantas *et al.* (2009), Moglia *et al.* (2010), Eudes *et al.* (2011), and Wang *et al.* (2011)
C4H	Cinnamate-4-hydroxylase	Poplar hybrid (*P. trichocarpa x P. deloides*), *Arabidopsis thaliana*, *Helianthus tuberosus*, *Glycine max*	Ro & Douglas (2004), Leonard *et al.* (2005), Yan *et al.* (2005), Vannelli *et al.* (2007), and Trantas *et al.* (2009)
CHI	Chalcone isomerase	*Petunia hybrid, Glycine max, Medicago sativa*	Leonard *et al.* (2005), Ralston *et al.* (2005), Yan *et al.* (2005, 2007), and Trantas *et al.* (2009)
CHR	Chalcone reductase	*Medicago sativa*	Yan *et al.* (2007)
CHS	Chalcone synthase	*Petunia hybrid, Medicago sativa, Hypericum androsaemum, Glycine max*	Jiang *et al.* (2005), Leonard *et al.* (2005), Yan *et al.* (2005, 2007), and Trantas *et al.* (2009)
F3H	Flavanone 3β-hydroxylase	*Glycine max*	Ralston *et al.* (2005), and Trantas *et al.* (2009)
F3'H	Flavonoid 3-hydroxylase	*Glycine max*	Trantas *et al.* (2009)
FLS	Flavonol synthase	*Solanum tuberosum*	Trantas *et al.* (2009)
FSI	Flavone synthase I	*Petroselinum crispum*	Leonard *et al.* (2005)
FSII	Flavone synthase II	*Glycine max*, *Antirrhinum majus*	Leonard *et al.* (2005), and Ralston *et al.* (2005)
HCBT	Hydroxycinnamoyl/benzoyl-CoA/ anthranilate *N*–hydroycinnamoyl/ benzoyltransferase	*Dianthus caryophyllus*	Eudes *et al.* (2011)
HCT	Hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase	*Cynara cardunculus*	Moglia *et al.* (2010)
IFS	Isoflavone synthase	*Glycine max*	Ralston *et al.* (2005), and Trantas *et al.* (2009)
PAL/TAL	Phenylalanine ammonia lyase/Tyrosine ammonia lyase	Poplar hybrid (*P. trichocarpa x P. deloides*)/*Rhodobacter sphaeroides*, *Rhodosporidium toruloides*	Ro & Douglas (2004)/Jiang *et al.* (2005), Vannelli *et al.* (2007), Trantas *et al.* (2009), and Wang *et al.* (2011)
STS	Stilbene synthase	*Vitis vinifera*	Becker *et al.* (2003), Beekwilder *et al.* (2006), Zhang *et al.* (2006), Trantas *et al.* (2009), and Wang *et al.* (2011)

Table 2. Isoprenoid biosynthetic genes (both heterologous and native) used to build production pathways in yeast

Type	Name	Species	Publications
Pyrophosphate synthase	*ERG20* (ergosterol biosynthesis 20)	*Saccharomyces cerevisiae*	Ro *et al.* (2006, 2008) and Ohto *et al.* (2010)
	FPPS (farnesyl pyrophosphate synthase)	*Arabidopsis thaliana*	Farhi *et al.* (2011)
	BTS1 (bet two suppressor)	*Saccharomyces cerevisiae*	Verwaal *et al.* (2007), Tokuhiro *et al.* (2009), Ukibe *et al.* (2009), and Ohto *et al.* (2010)
	crtE (carotenoid biosynthetic gene E)	*Xanthophyllomyces dendrorhous, Erwinia uredovora*	Yamano *et al.* (1994), Miura *et al.* (1998), and Verwaal *et al.* (2007)
	GGPPS (geranylgeranyl pyrophosphate synthase)	*Taxus chinensis, Sulfolobus acidocaldarius, Taxus canadensis*	Dejong *et al.* (2006) and Engels *et al.* (2008)
Isoprenoid synthase	*GES* (geraniol synthase)	*Ocimum basilicum*	Oswald *et al.* (2007) and Fischer *et al.* (2011)
	LIS (linalool synthase)	*Clarkia breweri*	Herrero *et al.* (2008), and Rico *et al.* (2010)
	ADS (amorphadiene synthase)	*Artemisia annua*	Ro *et al.* (2006), Paradise *et al.* (2008), Ro *et al.* (2008), and Farhi *et al.* (2011)
	Cubebol synthase	*Citrus × paradisi*	Asadollahi *et al.* (2008, 2009, 2010)
	Epicedrol synthase	*Artemisia annua*	Jackson *et al.* (2003)
	Patchoulol synthase	*Pogostemon patchouly*	Asadollahi *et al.* (2008)
	Valencene synthase	*Citrus × paradise, Citrus sinensis*	Asadollahi *et al.* (2008) and Farhi *et al.* (2011)
	TS (taxadiene synthase)	*Taxus chiensis, Taxus brevifolia*	Dejong *et al.* (2006) and Engels *et al.* (2008)
Isoprenoid-modifying cytochrome P450*	*CYP71AV1* (amorphadiene oxidase)	*Aretmisia annua*	Ro *et al.* (2006, 2008)
	THY5α (taxadiene 5α hydroxylase)	*Taxus cuspidata*	Dejong *et al.* (2006) and Rontein *et al.* (2008)
	THY10b (taxoid 10β-hydroxylase)	*Taxus cuspidata*	Jennewein *et al.* (2005)
	CYP11A1 (steroid side chain cleavage)	*Bos taurus*	Duport *et al.* (1998, 2003) and Szczebara *et al.* (2003)
	CYP11B1 (11β-steroid hydroxylase)	*Bos taurus, Homo sapiens*	Dumas *et al.* (1996) and Szczebara *et al.* (2003)
	CYP17A1 (17α-steroid hydroxylase)	*Bos taurus*	Szczebara *et al.* (2003)
	CYP21A1 (21-steroid hydroxylase)	*Homo sapiens*	Szczebara *et al.* (2003)
Isoprenoid-modifying other	*DPP1* (diacylglycerol diphosphate phosphatase)	*Saccharomyces cerevisiae*	Tokuhiro *et al.* (2009)
	Δ7-sterol reductase	*Arabidopsis thaliana*	Duport *et al.* (1998, 2003) and Szczebara *et al.* (2003)
	3β-HSD (3β- hydroxyl steroid dehydrogenase/isomerase)	*Bos taurus, Homo sapiens*	Duport *et al.* (1998) and Szczebara *et al.* (2003)
Mevalonate pathway rate-controlling enzyme	*tHMG1* (HMG-CoA reductase truncated), or *HMG1* (wild-type HMG-CoA reductase)	*Saccharomyces cerevisiae*	Ro *et al.* (2006), Engels *et al.* (2008), Ro *et al.* (2008), Tokuhiro *et al.* (2009), Asadollahi *et al.* (2010), Ohto *et al.* (2010), Rico *et al.* (2010), and Farhi *et al.* (2011)
Ergosterol pathway transcription factor	*upc2-1* (uptake control 2)	*Saccharomyces cerevisiae*	Jackson *et al.* (2003), Ro *et al.* (2006), Engels *et al.* (2008), and Ro *et al.* (2008)
Carotenoid biosynthetic genes	*crtY, crtI,crtS, crtR, crtW, crtZ, crtB*	*Xanthophyllomyces dendrorhous, Paracoccus* sp., *Pantoea ananatis, Erwinia uredovora, Agrobacterium aurantiacum*	Yamano *et al.* (1994), Miura *et al.* (1998), Verwaal *et al.* (2007), Ukibe *et al.* (2009), and Verwaal *et al.* (2010)
Pyruvate dehydrogenase bypass enzymes	*ACS1* (acetyl-CoA synthase 1)	*Saccharomyces cerevisiae*	Shiba *et al.* (2007)
	ALD6 (acetaldehyde dehydrogenase 6)	*Saccharomyces cerevisiae*	Shiba *et al.* (2007)

*Isoprenoid-modifying cytochrome P450s were expressed together with heterologous partner redox proteins, namely cytochrome P450 reductase partners (CPR) for sesquiterpenes and diterpenes, adrenodoxin reductase (*ADR*) and adrenodoxin (*ADX*) for steroids.

Table 3. Alkaloid biosynthetic genes used to build production pathways in yeast

Abbreviation	Gene name	Species	Publications
4'OMT	3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase	*Thalictrum flavum, Papaver somniferum*	Hawkins & Smolke (2008)
6'OMT	Norcoclaurine 6-*O*-methyltransferase	*Thalictrum flavum, Papaver somniferum*	Hawkins & Smolke (2008)
BBE	Berberine bridge enzyme	*Coptis japonica, Papaver somniferum*	Hawkins & Smolke (2008) and Minami *et al.* (2008)
CNMT	Coclaurine *N*-methyltransferase	*Coptis japonica, Thalictrum flavum, Papaver somniferum*	Hawkins & Smolke (2008) and Minami *et al.* (2008)
CYP2D6	Cytochrome P450 2D6	*Homo sapiens*	Hawkins & Smolke (2008)
CYP719A	Canadine synthase	*Thalictrum flavum*	Hawkins & Smolke (2008)
CYP80G2	Corytuberine synthase	*Coptis japonica*	Minami *et al.* (2008)
SGR	Strictosidine β-glucosidase	*Catharanthus roseus*	Geerlings *et al.* (2001)
SMT	(S)-scoulerine 9-*O*-methyltransferase	*Thalictrum flavum*	Hawkins & Smolke (2008)
STR	Strictosidine synthase	*Catharanthus roseus*	Geerlings *et al.* (2001)

Table 4. Polyketide biosynthetic genes used to build production pathways in yeast

Abbreviation	Gene name	Species	Publications
6-MSAS	6-methylsalicylic acid synthase	*Penicillium patulum*	Kealey *et al.* (1998), Wattanachaisaereekul *et al.* (2007, 2008)
ACC1	Acetyl-CoA carboxylase	*Saccharomyces cerevisiae*	Wattanachaisaereekul *et al.* (2008)
DEBS	6-deoxyerythronolide synthase (module 2)	*Saccharopolyspora erythraea*	Mutka *et al.* (2006)
PCC	Propionyl-CoA carboxylase	*Streptomyces coelicolor*	Mutka *et al.* (2006)
PPTase	Phosphopantetheinly transferase	*Bacillus subtilis, Aspergillus nidulans*	Kealey *et al.* (1998), Wattanachaisaereekul *et al.* (2007, 2008)
PrpE	Propionyl-CoA synthase	*Salmonella typhimurium*	Mutka *et al.* (2006)

Table 1 Example of products and strains of *S. cerevisiae*

Categories	Products	Specific applications	Strains	References
Biofuels	Ethanol	Redox balance problem by inhibiting glycerol formation in anaerobic culture was solved by combining gene deletion (*GPD1* and *GPD2*) and integration (*mhpF* from *E. coli*) with acetic acid supplementation, which was presented at substantial quantities in lignocellulosic hydrolysates of agricultural residues	CEN.PK102-3A (*MATa ura3 leu2*)	[17]
	Biobutanol	Overexpression of genes in valine metabolism, *ILV2*, *ILV3*, *ILV5*, and *BAT2* showed an increased production of isobutanol in *S. cerevisiae*, which strain was decided as a host because of relative tolerance to alcohols, and robustness in industrial fermentation	CEN.PK 2-1C (*MATα leu2-3, 112 his3-Δ1 ura3-52 trp1-289 MAL2-8(Con) MAL3 SUC3*)	[18]
	Biodiesels	Glycerol utilization for production of fatty acid ethyl esters (FAEEs) was done by amplification of ethanol production pathway, which is used for the transesterification in FAEEs synthesis, with overexpression of an unspecific acyltransferase from *Acinetobacter baylyi*	YPH499 (*MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-D63 his3-D200 leu2-D1*)	[19]
	Bisabolene (D2 diesel fuel, bisabolane)	Bisabolene, the immediate precursor to bisabolane, was produced by (1) using the strategy for increasing pool of farnesyl diphosphate (FPP) in artemisinic acid production [20] and (2) screening and codon-optimizing bisabolene synthases (sesquiterpene synthases). The final titers were over 900 mg/l in shake flasks	BY4742 (*MATα his3D1 leu2D0 lys2D0 ura3D0*)	[21]

Bulk chemicals	1,2-propanediol	The combination effects of different copy number (from 0 to 3) of two *E. coli* genes (*mgs* and *gldA*) ware studied. Although the three copy numbers of two genes showed the highest level of 1,2-propanediol, specific activity of Mgs and inhibitory relationship by GldA was considered more importantly for the production of 1,2-propanediol	NOY386αA (*MATα ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1*) BWG1-7a (*MATa ade1-100 his4-519 leu2-3,112 ura3-52 GAL*$^{+}$)	[22]
	D-ribose and ribitol	The flux from glucose to pentose phosphate pathway was amplified by inactivation of both phosphoglucose isomerase and transketolase with overexpression of sugar phosphate phosphatase (DOG1). Fructose was supplied and redox balance was controlled by overexpression of NAD+-specific glutamate dehydrogenase (*GDH2*) of *S. cerevisiae* or NADPH-utilizing glyceraldehyde-3-phosphate dehydrogenase (*gapB*) of *Bacillus subtilis*	CEN.PK2-1D (*VW-1B; MATα, leu2-3/112 ura3-52 trp1-289 his3Δ1 MAL2-8c SUC2*)	[23]
	L-lactic acid	Improved production of L-lactic acid was achieved by overexpression of *LDH* gene coding L-lactic acid dehydrogenase from bovine and knocked out a *PDC1* gene coding pyruvate decarboxylase to redirect the fluxes to L-lactic acid; and overexpression of an NADH oxidase (*nox*) from *Streptococcus pneumoniae* into the cytoplasm to reduce the ratio of NADH/NAD+	CEN. PK2-1C (*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*)	[24]
	Polyhydroxy-alkanoates	The synthesis of diverse size of PHA polymer (C4 to C14) was investigated by cytosolic expression of mcl-PHA synthase from *Pseudomonas oleovorans* or peroxisomal expression of scl-PHA synthase from *Ralstonia eutropha*	BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0*)	[25]
	Pyruvic acid	Pyruvate decarboxylase-negative [Pdc(−)] strains were evolved in glucose-limited chemostat cultivation by progressively lowering the acetate content in the feed to obtain an acetate-independent Pdc (−) mutant. Maximum yield was 0.54 g of pyruvate/g glucose	CEN.PK113-7D (*MATa MAL2-8C, SUC2*)	[26]
	Succinic acid	The deletion of the genes *SDH1, SDH2, IDH1*, and *IDP1* made higher flux to succinic acid production. Maximum yield was 0.11 mol of succinic acid/mol of glucose	AH22ura3 (*MATa ura3Δ leu2-3 leu2-112 his4-519 can1*)	[27]

Fine chemicals	β-amyrin	The differences of phenotype and genotype in two yeast strains, CEN.PK113-7D and S288C, were compared. CEN.PK113-7D had more contents of ergosterol and fatty acids with non-silent SNPs in relative metabolism, *ERG8, ERG9*, and *HFA1*. Amplification of those genes exhibited a fivefold increase of β-amyrin	CEN.PK113-7D (*MATa MAL2-8C SUC2*)
	β-carotene	Genomic integration and overexpression of carotenogenic genes from *X. dendrorhous* (*crtYB, crtE*, and *crtI*) and *S. cerevisiae* (*BTS1* and truncated *HMG1*) with change of copy number achieved high levels of β-carotene, up to 5.9 mg/g dry cell weight	CEN.PK113-7D (*MATa MAL2-8C SUC2*)
	Amorpha-4, 11- diene	Amplification of mevalonate pathway in CEN.PK2 was engineered and compared to previously constructed strain S288C [20]. Artemisinic acid production was doubled, while amorpha-4, 11-diene was tenfold higher, over 40 g/l	CEN.PK2-1C (*MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*) CEN.PK2-1D (*MATα ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2*)
	Valencene and amorphadiene	Co-expression of heterologous enzymes, farnesyl diphosphate synthases (FDPSs), and sesquiterpene synthase (ex. *Citrus sinensis* valencene synthase *CsTPS1*, *Artemisia annua* terpene synthase, amorpha-4,11-diene synthase ADS) in mitochondria and cytosol improved the production of valencene and amorphadiene	W303-1A (*MATa, ade2-1 trp1-1 leu2-3, 112 his3-11, 15 ura3-1*) mBDXe (a uracilauxotroph derivative of strain BDX, Lallemand, Rexdale, Ontario, Canada)
	Casbene (an anti-fungal diterpene)	Genes of putative Casbene synthases from different Euphorbiaceae species were isolated and applied for production of diterpenes. Maximum concentration of Casbene was 31 mg/l	BY4742 (*MATα his3D1 leu2D0 lys2D0 ura3D0*)
	Cinnamoyl anthranilates	Twenty-six different cinnamoyl anthranilates molecules were produced by co-expressing a 4-coumarate/CoA ligase (4CL, EC 6.2.1.12) from *Arabidopsis thaliana* and a hydroxycinnamoyl/benzoyl-CoA/anthranilate *N*-hydroxycinnamoyl/benzoyltransferase (HCBT, EC 2.3.1.144) from *Dianthus caryophyllus*	BY4742 (*MATα his3D1 leu2D0 lys2D0 ura3D0*)
	Cubebol	Overexpression of *GFTpsC* (a sesquiterpene synthase isolated from *Citrus paradisi* and encoding for a cubebol synthase) with integration of *tHMG1* into genome and reduction of *ERG9* gene expression produced cubebol up to 10 mg/l	CEN.PK113-5D (*MATa MAL2-8c SUC2 ura3-52*)

Eicosapentaenoic acid (EPA)	Five heterologous fatty acid desaturases and an elongase were identified by a BLAST search and assayed their substrate preferences activity. Without supplement of fatty acids, EPA/ARA were produced	CEN.PK113-5D (*MATa MAL2-8c SUC2 ura3-52*)
Farnese and geranyl geraniol	*ERG9* deletion and overexpression of two isozymes of HMGCoA reductases (*HMG1* and *HMG2*) was implemented in a host strain with overexpression of diverse FPP synthases and GGPP synthases	FL100 (*MATa*, ATCC: 28383)
L-ascorbic acid	About 100 mg of L-ascorbic acid per liter was produced by overexpression of D-arabionono-1,4-lactose oxidase from *S. cerevisiae* and L-galactose dehydrogenase from *Arabidopsis thaliana*	GRF18U (*MATα his3 leu2 ura3; NRRL Y-30320*) W303 1B (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*)
Linalool	Overexpression of *Clarkia breweri* linalool synthase gene (LIS) in wine strain T_{73} showed higher levels of linalool than conventional laboratory strains. Combining with deregulation of HMG-CoA reductase improved linalool yield	BQS252 (*MATa ura3-52* (derivative of FY1679))
Methylmalonyl-coenzyme A	Polyketide precursor (Methylmalonyl-CoA) pathway was constructed by introducing propionyl-CoA carboxylase and malonyl/methylmalonyl-CoA ligase from *Streptomyces coelicolor*	InvSC1 (*MATa, his3delta1, leu2, trp1-289, ura3-52* (Invitrogen, Carlsbad, CA, USA)) BJ5464 (*MATα, ura3-52, trp1, leu2-delta1, his3-delta200, pep4::HIS3, prb1-delta1.6R, can1, GAL*).

Patchoulol	A physical fusion between native (farnesyl diphosphate synthase) and heterologous enzymes (patchoulol synthase of plant origin, *Pogostemon cablin* was successfully applied to produce patchoulol, 25 mg/l	CEN.LA100 (*MATa/MATα ERG20/erg20::hph MAL2-8c/ MAL2-8c SUC2/SUC2 ura3-52/ ura3-52*)
Resveratrol	Co-expression of the coenzyme-A ligase-encoding gene (*4CL216*) from a hybrid poplar and the grapevine resveratrol synthase gene (*vst1*) from *Vitis vinifera* with supplement of *p*-coumaric acid produced resveratrol, 1.45 mg/L	FY23 (*MATa ura3-52 trplA63 leu2A1*)
Vanillin	Knock-out targets, *PDC1* and *GDH1*, suggested by in silico metabolic model was applied and production of vanillin was improved up to fivefold	X2180-1A (*MATa his3D1 leu2D0 met15D0 ura3D0 adh6::LEU2 bgl1::KanMX4 PTPI1::3DSD [AurC]::HsOMT [NatMX]::ACAR [HphMX]*)
Se-methylse-lenocysteine	Combination of metabolic (codon optimization of heterologous selenocysteine methyltransferase) and bioprocess (tuning carbon-and sulfate-limited fed-batch) engineering achieved 24-fold increase in Se-methylselenocysteine production	CEN.PK113-7D (*MATa MAL2-8C SUC2*)
Non-ribosomal peptides	Separated non-ribosomal peptide synthetase modules with compatible communication-mediating domains showed functional interaction, which meant that new module combinations could produce novel non-ribosomal peptides	CEN.PK113-11C (*MAT a MAL2-8c SUC2 ura3-52 his3-D1*)

Protein drugs	Insulin-like growth factor 1 (fhlGF-1)	Inactivation of *GAS1* increased the yield of human insulin-like growth factor1, from 8 to 55 mg/l	GcP3 (*MAT a pep4-3 prb1-1122 ura3-52 leu2 gal2 cir°*)
	Glucagon	Disruption of *YPS1* encoded aspartic protease increased glucagon, 17.5 mg/l	SY107 (*MATα YPS1 Δtpi::LEU2 pep4-3 leu2 Δura3 cir$^+$*)
	Single-chain antibodies (scFv)	Production of an anti-transferrin receptor single-chain antibody (OX26 scFv) was optimized by adjusting expression temperature and gene dosage and final yield was 0.5 mg/l	BJ5464 (*MATa ura3-52 trp1 leu2D1 his3D200 pep40HIS3 prb1D1.6R can1 GAL*)
	Hepatitis surface antigen (HBsAg)	Glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter of *Pichia pastoris* was used for HBsAg production and final yield was 19.4 mg/l	INVSc1 (*MATa his3D1 leu2 trp1-289 ura3-52*)
	Parvovirus B19 VP2	The major-capsid protein VP2 of Parvovirus B19 produced in *S. cerevisiae* showed similar properties to native virus or produced by baculovirus system in size, molecular weight, and antigenicity. The yield was 400 mg/l	HT393 (*MATa leu2-3 leu2-112 ura3Δ5 prb1-1 prc1-1 pra1-1 pre1-1*)
	Epidermal growth factor (EGF)	*O*-glycosylation pathway was constructed by introduction of GFR (GDP-fucose transporter), POFUT1 (*O*-fucosyltransferase 1), *manic fringe* gene (β1,3-*N*-acetylglucosaminyltransferase) from human and *MUR1* (GDP-mannose-4,6-dehydratase), *AtFX/GER1*(GDP-4-keto-6-deoxy-mannose-3,5-epimerase/4-reductase) from *Arabidopsis thaliana* producing *O*-glycosylated EGF protein	W303-1A (*MATa leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100*) W303-1B (*MATα leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100*)
	Immunoglobulin G	Leader peptides for the enhanced secretion of proteins constructed by directed evolution allowed for a 180-fold increase in secretion of full-length, functional, glycosylated human IgG	BJ5464a (*MATα ura3-52 leu2∼1 his3∼200 pep4::HIS3 prb1∼1.6Rcan1 GAL*)
	Hepatitis B virus surface antigen (HBsAg)	The yield of S domain of hepatitis B virus surface antigen (sHBsAg) was increased by co-expression of disulfide isomerase (*PDI1*) with adjusting fermentation mode	*S. cerevisiae* 2805 (*MATα pep4::HIS3 prb-Δ1.6 his3 ura3-52 gal2 can1*)
	L1 protein of human papillomavirus (HPV) type16	Optimization of the secondary structure of HPV16 L1 mRNA increased the expression level of that protein up to fourfold than of wild-type	*S. cerevisiae* 2805 (*MATα pep4::HIS3 prb-Δ1.6 his3 ura3-52 gal2 can1*)

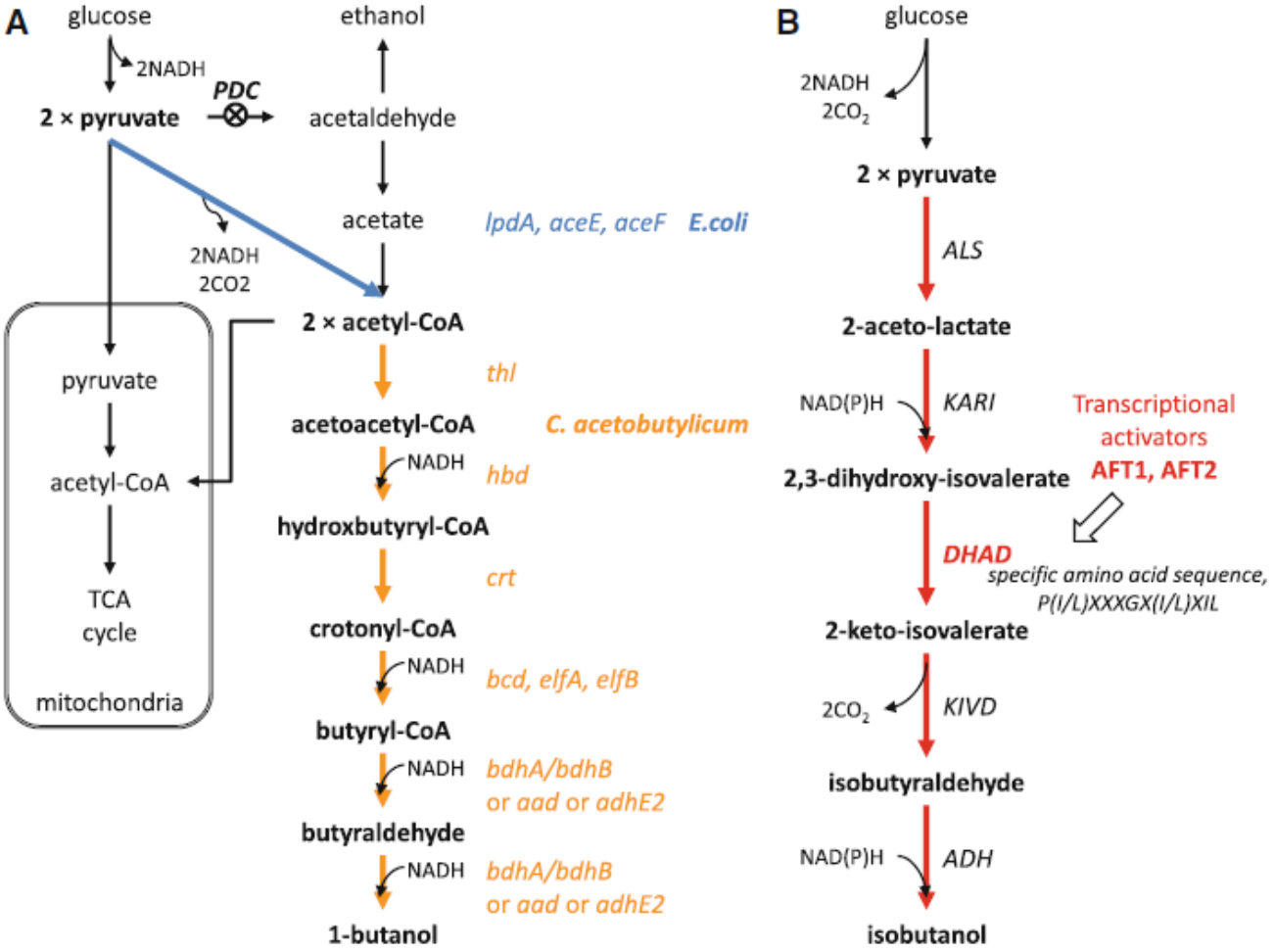


Fig. 5 Illustration of Gevo's strategies for *n*-butanol and isobutanol production in the cytosol [90, 91, 105]. **a** *n*-butanol production was attempted by amplification of heterologous genes such as the pyruvate dehydrogenase multienzyme complex (*lpdA, aceE, aceF*) from *E. coli* for increasing the cytosolic acetyl-CoA pool, and the genes in butanol synthetic pathway from *Clostridia* species. Moreover, the activity of pyruvate decarboxylase (PDC) was reduced. **b** Isobutanol was produced in the cytosol to avoid cofactor balancing in the mitochondria; all the genes in isobutanol pathway were over-expressed in cytosol. Especially, dihydroxyacid dehydratases (DHAD) from *Lactococcus lactis* and *Neurospora crassa* were used, which had specific amino sequence, P(I/L)XXXGX(I/L)XIL. Also, the transcriptional activators *AFT1/AFT2* were over-expressed to increase DHAD activity

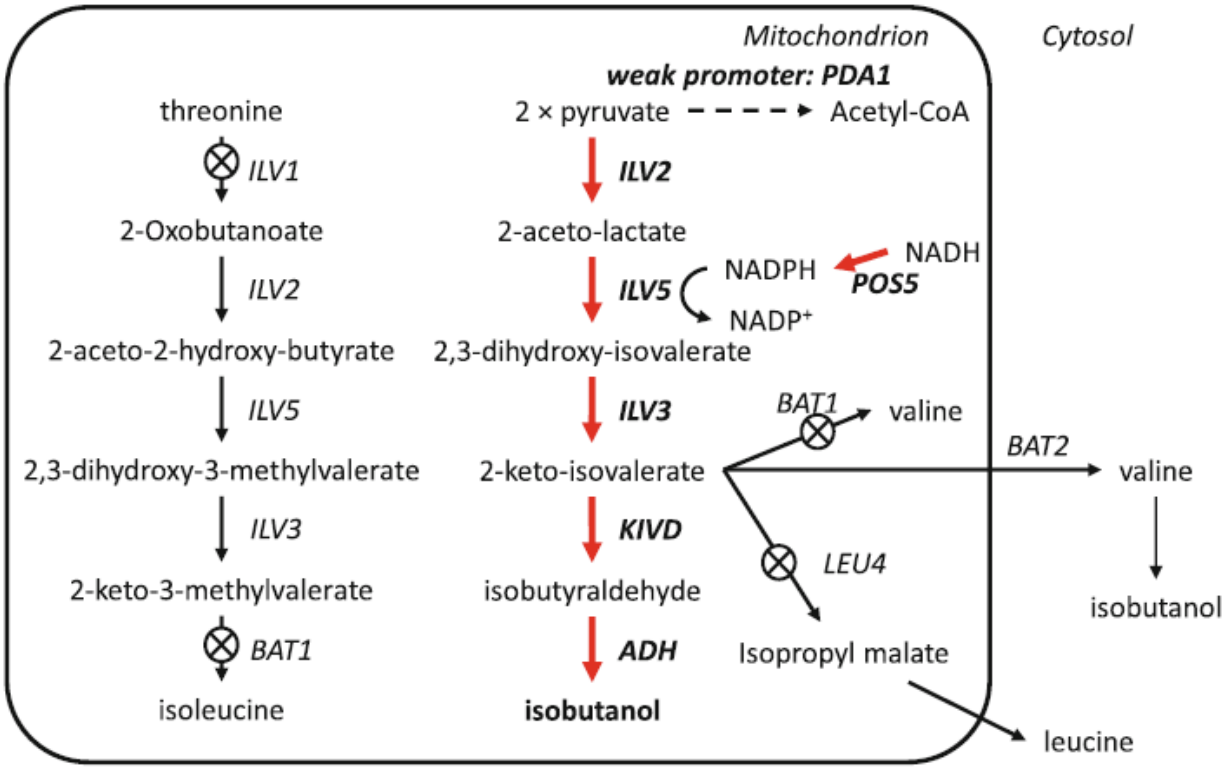


Fig. 8 Butamax's isobutanol production strategies in the mitochondria [103]. To block substrate-competing reactions *BAT1*, *ILV1*, and *LEU4* were deleted and the activity of the E1 alpha subunit of the pyruvate dehydrogenase (*PDH*) complex (*PDA1*) was reduced by promoter exchange to a weak one. NADH kinase (*POS5*) was over-expressed to ensure sufficient supply of NADPH required by the KARI enzyme. *Red arrows* mean over-expression of genes

Table 4 Targets for increasing butanol tolerance in yeast (Butamax)

Targeting	Modified genes	Butanol tolerance [growth yield improvement in butanol % (w/v)]	References
Multidrug resistance ATP-binding cassette transporter	Pdr5p, CDR1, BFR1	~1.8-fold in 0.75%	[114]
Cell wall integrity pathway	SLT2p	~25% in 1%	[115]
Osmolality/glycerol response pathway	PBS2p	~40% in 1%	[116]
Filamentous growth response pathway	MSS11p	~2-fold in 1.5%	[117]
Amino acid starvation	Gcn1p, Gcn2p, Gcn3p, Gcn4p, Gcn5p, Gcn20p	~1.8-fold in 2.0%	[118]

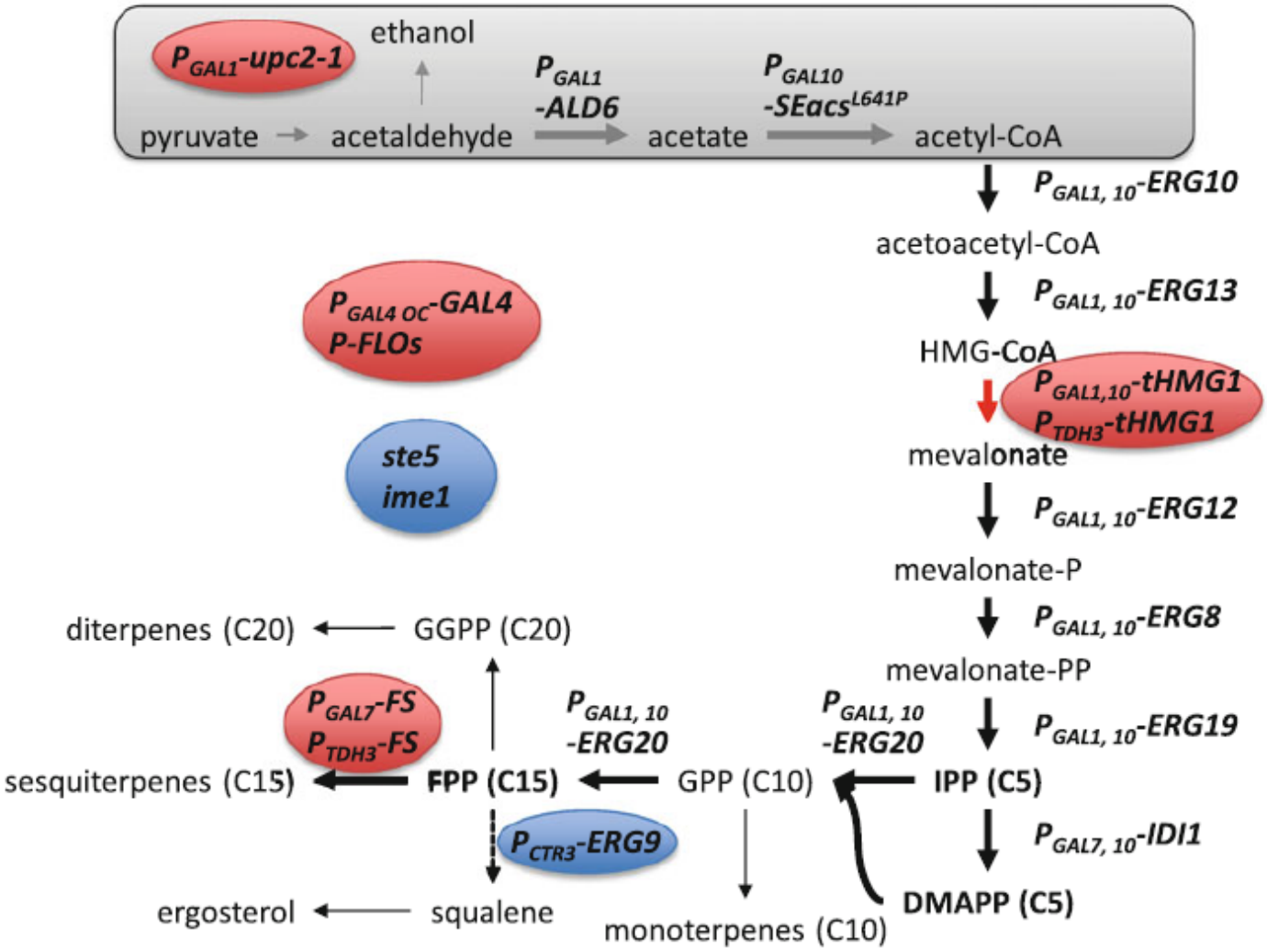


Fig. 9 Overview of Amyris metabolic engineering strategies. Industrial strain *Saccharomyces cerevisiae PE-2* was used as a production host because of its higher tolerance to the industrial environment [129]. All promoters of mevalonate genes were exchanged to strong one in chromosome. *Gray box* means the strategies that were used in a scientific article [124] but not in the patent. *Red color circles* mean even higher expression than other overexpressed genes. *Blue color circles* mean knock-out of genes or reduction of expression level. *Thick arrows* mean amplified steps based on plasmids in a scientific article [20]. The *dotted arrow* indicates reduction of flux

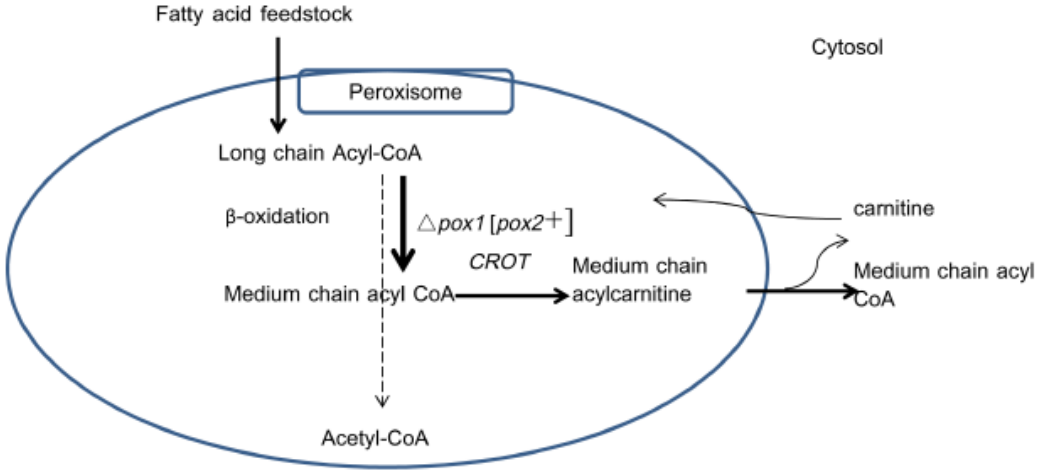


Figure 1. Genetic modification of the β-oxidation pathway in *Saccharomyces cerevisiae*. The dashed line represents the original pathway; the solid line represents the modified pathway. The only acyl-CoA oxidase (encoded by the gene *POX1*) in the *S. cerevisiae* genome was deleted, and the *POX2* gene from *Yarrowia lipolytica*, which encodes acyl-CoA oxidase with a preference for long chain acyl-CoAs, was expressed. To unblock the β-oxidation pathway, peroxisomal carnitine octanoyltransferase (*CROT*) from *Mus musculus* was also expressed to transport medium chain fatty acyl-CoAs out of peroxisomes.
doi:10.1371/journal.pone.0084853.g001

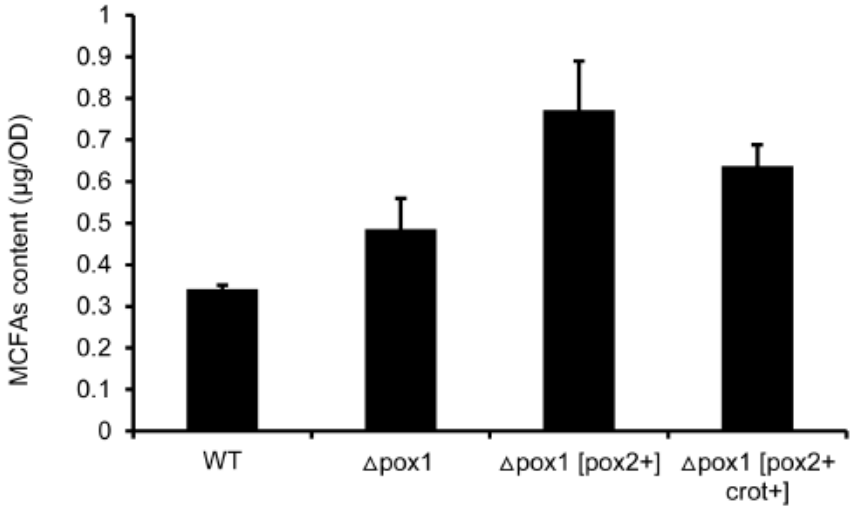
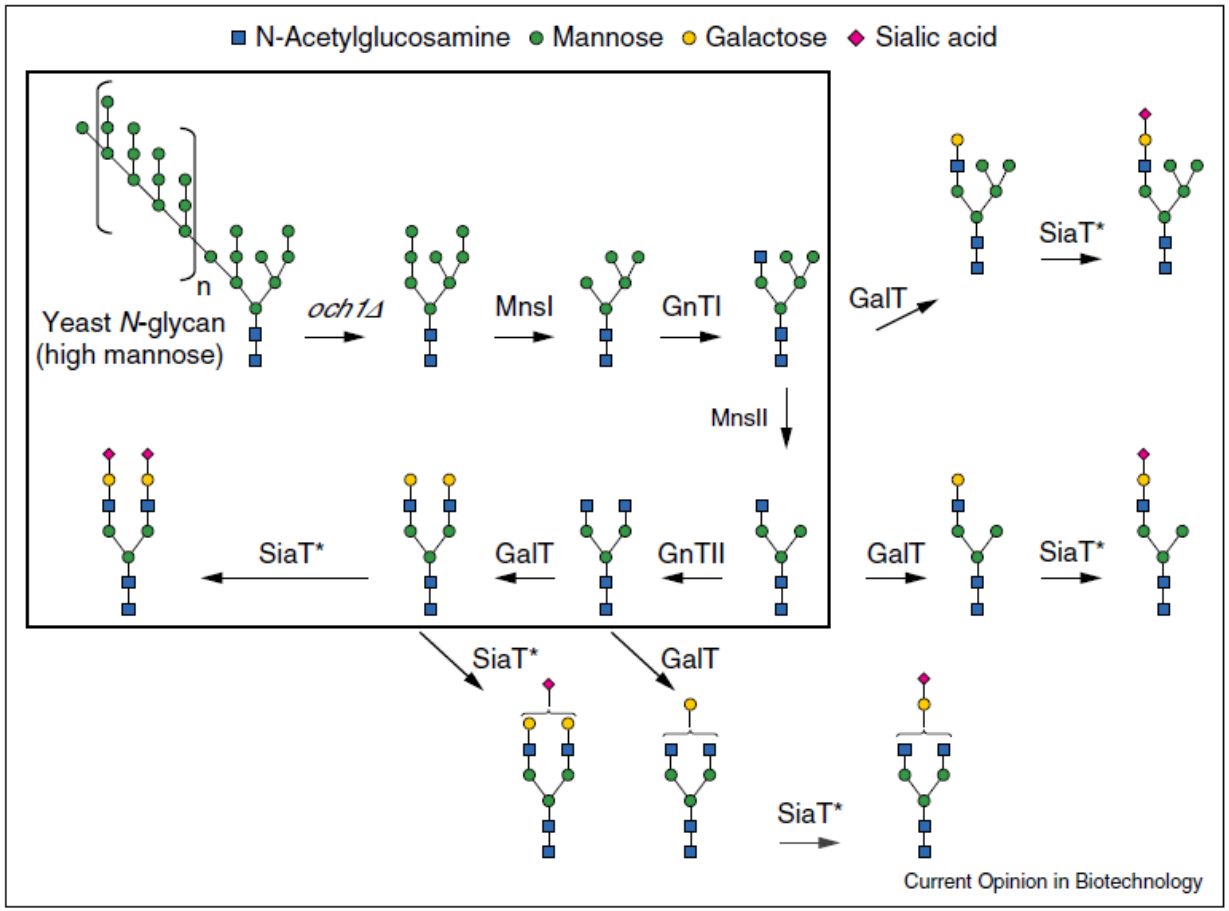


Table 1. Fatty acid production in the cell extract of the WT and the engineered strains[a].

Fatty acid type	WT	Δ*pox1*	Δ*pox1* [*pox2+*]	Δ*pox1*[*pox2+ crot+*]
C12:0	0.341	0.486	0.772	0.637
C14:0	1.183	1.590	2.143	1.855
C16:1	0.881	1.384	1.878	1.788
C16:0	30.623	27.675	35.794	32.535
C18:1	1.449	2.977	3.334	3.170
C18:0	24.889	22.959	32.890	28.548
C20:0	0.120	0.143	0.202	0.227
Total fatty acids	59.486	57.214	77.014	68.760

[a]Data represent fatty acid composition in μg/OD cell when WT (wild-type) and engineered strains were cultured in $YNBD_{0.5}O_3$ medium. The values are the means from three experiments examining the cell extracts at 24 h. The standard deviations were <5% of the values.
doi:10.1371/journal.pone.0084853.t001



Yeast *N*-glycan engineering. The *N*-glycosylation pathway of glycoengineered *Pichia pastoris* was previously reviewed [34]. Glycoproteins harboring predetermined glycoforms [78] are obtained depending on the glycoengineered yeast host used, each of which contains a unique set of gene deletions and glycosylation enzymes, as indicated by arrows. The main glycosylation pathway to obtain mammalian biantennary glycans is shown in the upper left rectangle. As indicated by (*), sialic acid linkages may be exclusively α-2,6 or α-2,3 depending on the chosen sialyltransferase. Other yeast modifications (*e.g.* beta-linked mannose, mannosylphosphate) are not depicted in the figure.

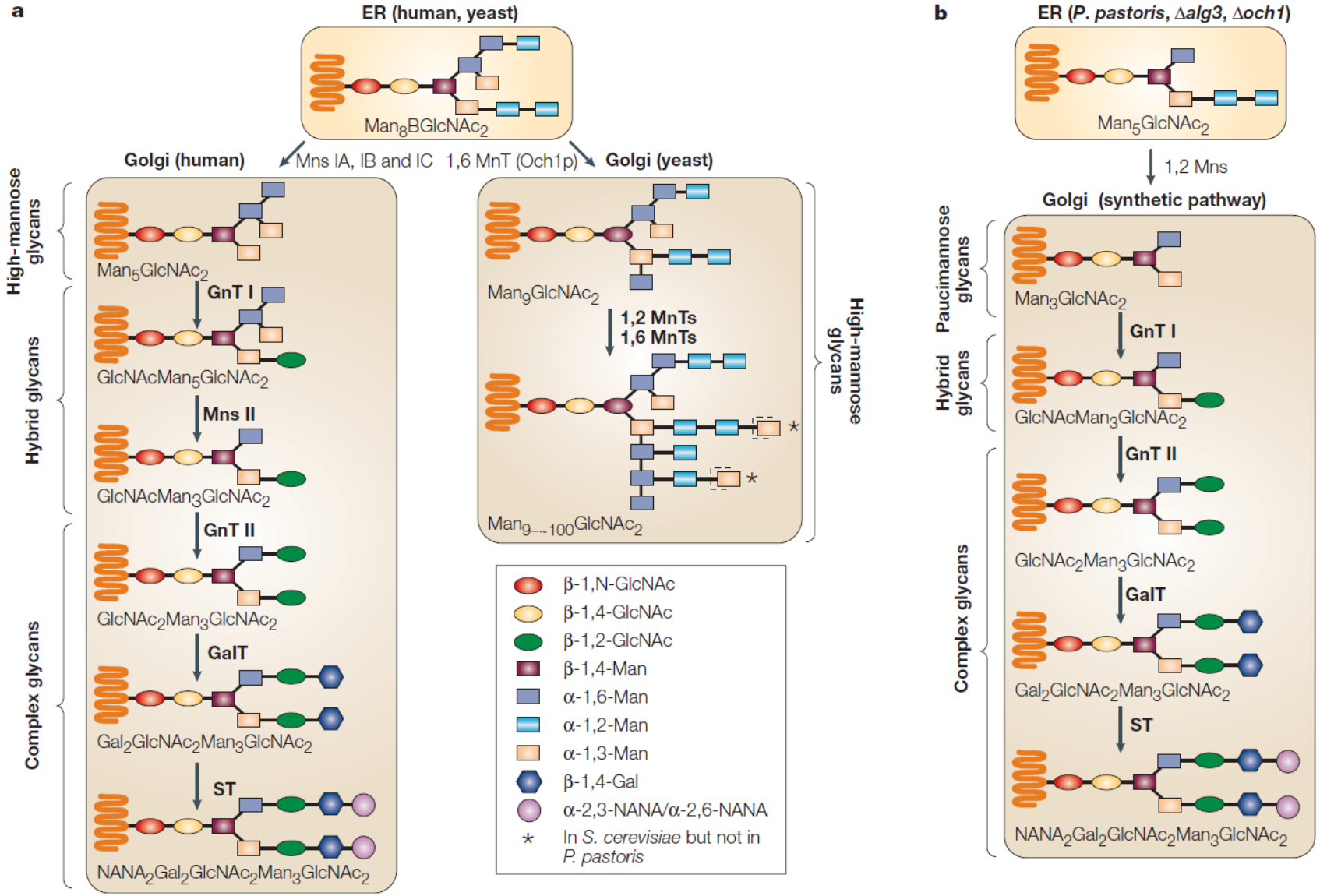
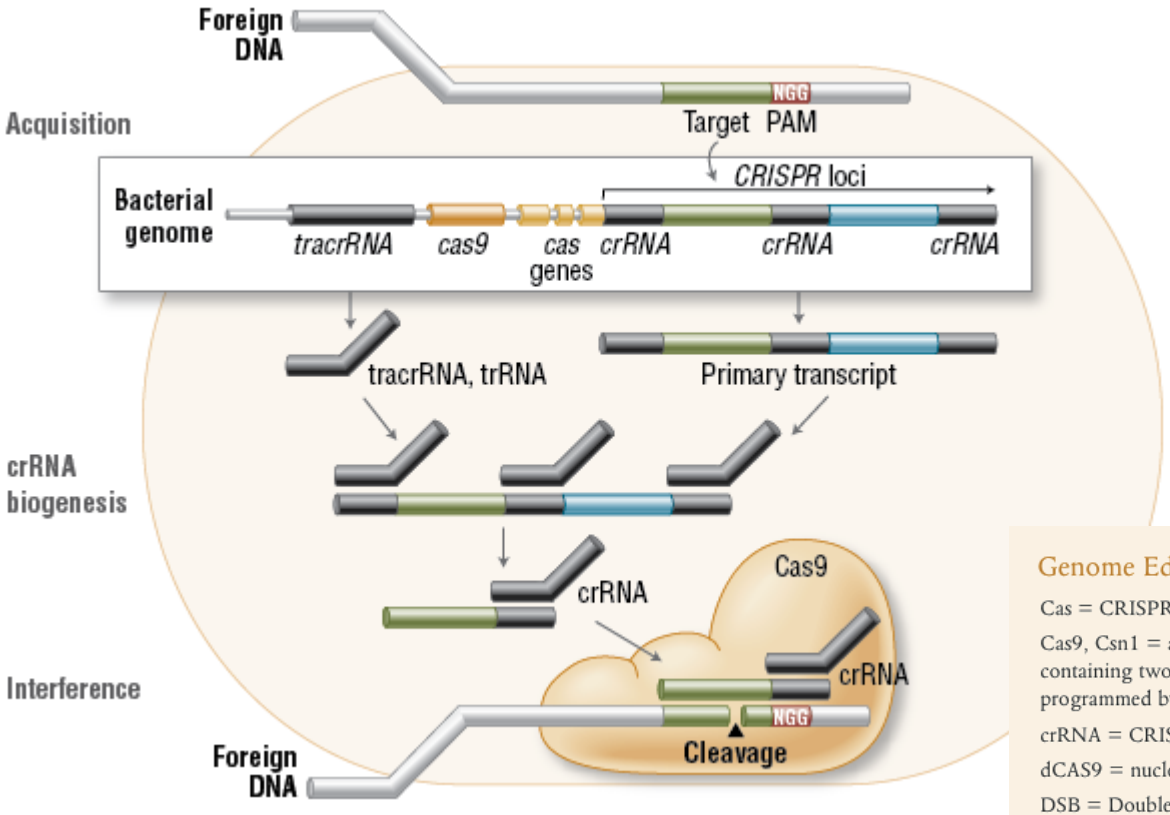


Figure 1 | **Major *N*-glycosylation pathways in humans and yeast. a** | Representative pathway of *N*-glycosylation pathways in humans (left) provides a template for humanizing *N*-glycosylation pathways in yeast (right). **b** | Early oligosaccharide assembly mutants can be used to recreate synthetic glycosylation pathways that lead to complex *N*-glycosylation in yeast (see main text). ER, endoplasmic reticulum; GalT, galactosyltransferase; GlcNAc, *N*-acetylglucosamine; GnT I, *N*-acetylglucosaminyl transferase I; GnT II, *N*-acetylglucosaminyl transferase II, Man, mannose; Mns II, mannosidase II; MnTs, mannosyltransferase; NANA, *N*-acetylneuraminic acid; ST, sialyltransferase.

CRISPR/Cas9 genome editing

- CRISPR is system of bacterial immunity



Genome Editing Glossary

Cas = CRISPR-associated genes

Cas9, Csn1 = a CRISPR-associated protein containing two nuclease domains, that is programmed by small RNAs to cleave DNA

crRNA = CRISPR RNA

dCAS9 = nuclease-deficient Cas9

DSB = Double-Stranded Break

gRNA = guide RNA

HDR = Homology-Directed Repair

HNH = an endonuclease domain named for characteristic histidine and asparagine residues

Indel = insertion and/or deletion

NHEJ = Non-Homologous End Joining

PAM = Protospacer-Adjacent Motif

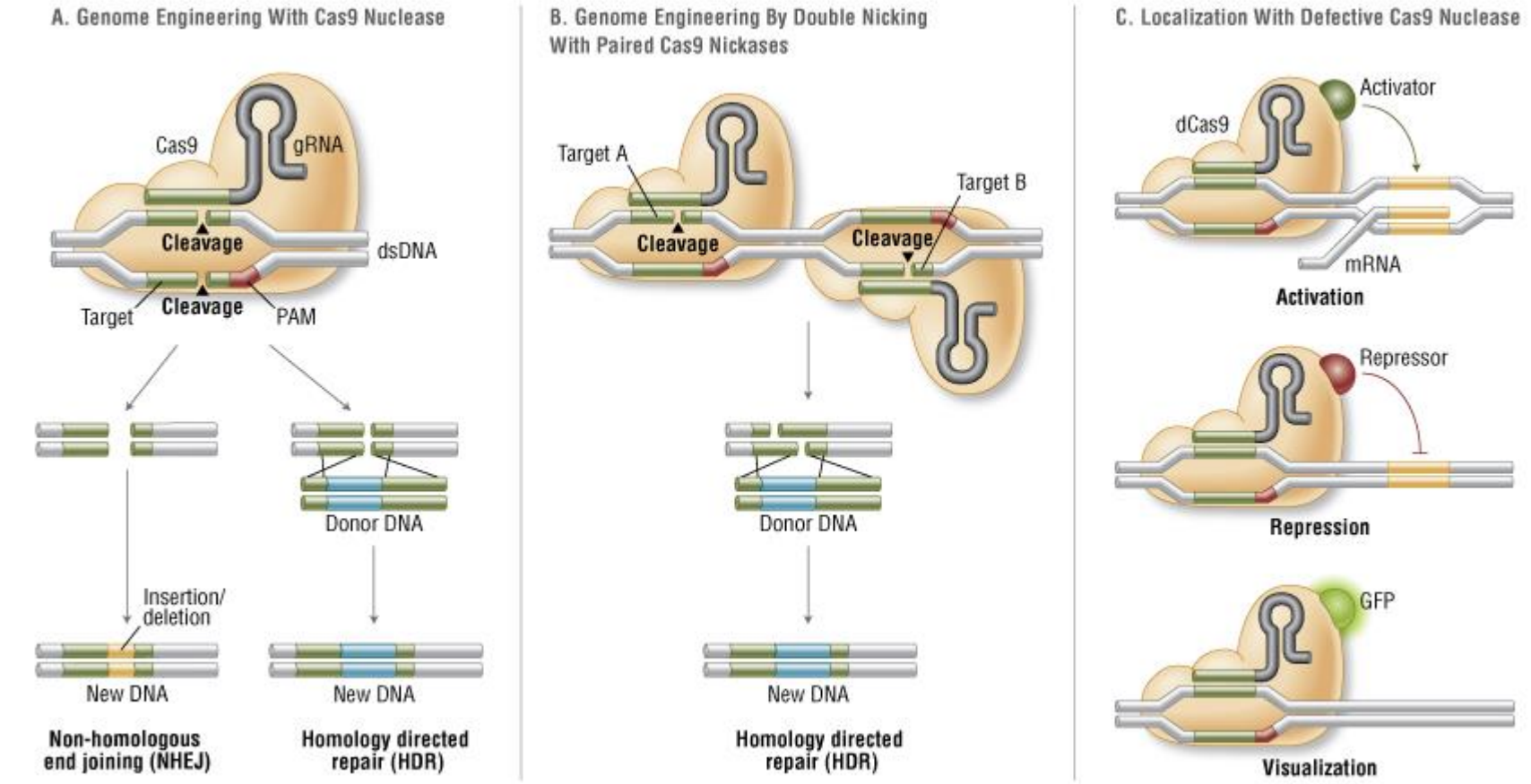
RuvC = an endonuclease domain named for an *E. coli* protein involved in DNA repair

sgRNA = single guide RNA

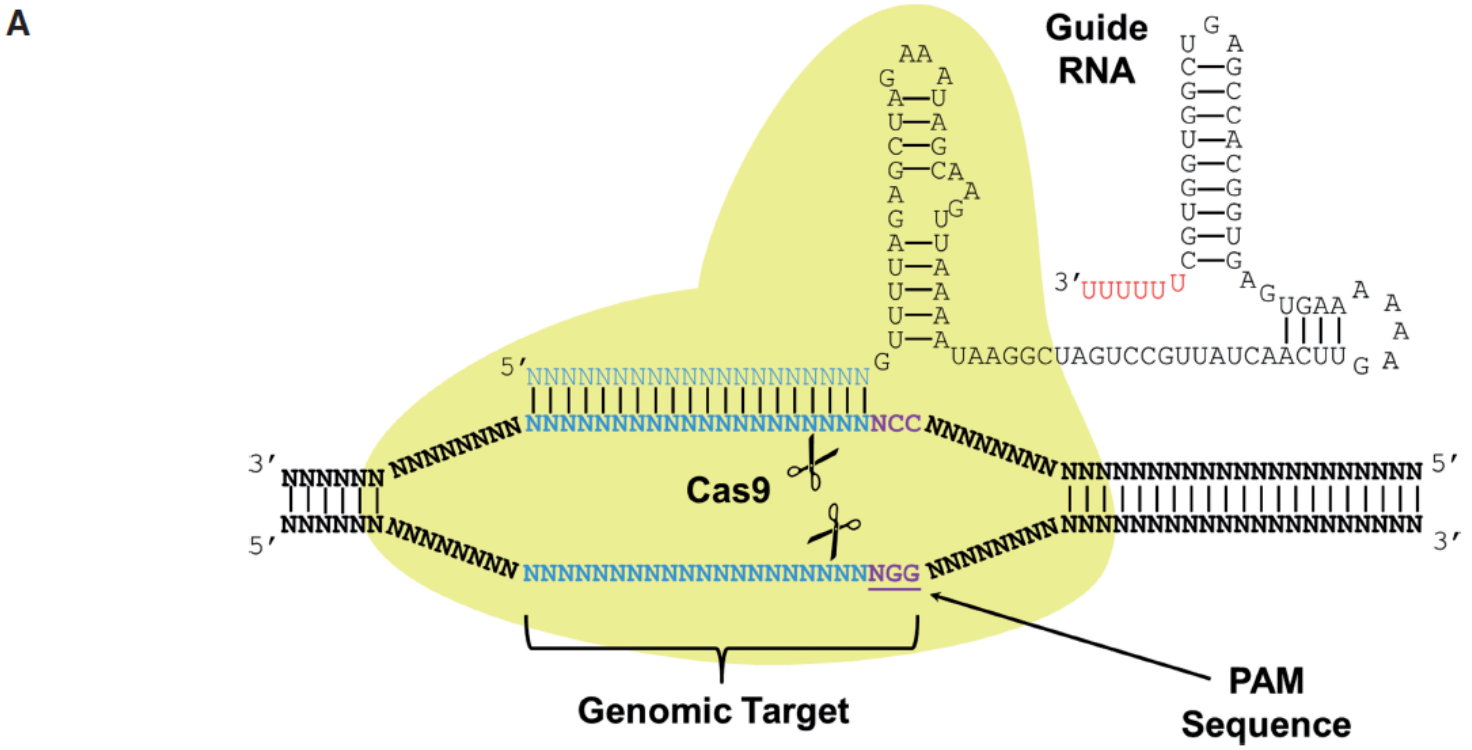
tracrRNA, trRNA = trans-activating crRNA

TALEN = Transcription-Activator Like Effector Nuclease

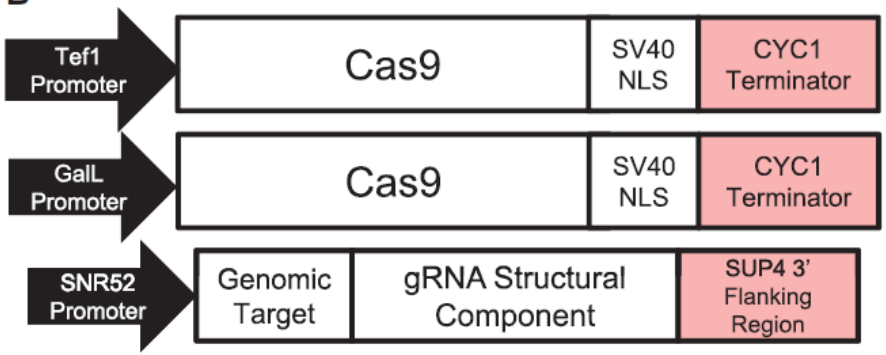
ZFN = Zinc-Finger Nuclease



A. Wild-type Cas9 nuclease site specifically cleaves double-stranded DNA activating double-strand break repair machinery. In the absence of a homologous repair template non-homologous end joining can result in indels disrupting the target sequence. Alternatively, precise mutations and knock-ins can be made by providing a homologous repair template and exploiting the homology directed repair pathway.
B. Mutated Cas9 makes a site specific single-strand nick. Two sgRNA can be used to introduce a staggered double-stranded break which can then undergo homology directed repair.
C. Nuclease-deficient Cas9 can be fused with various effector domains allowing specific localization. For example, transcriptional activators, repressors, and fluorescent proteins.



B



gRNA	Genomic Target	PAM
CAN1.Y	GATACGTTCTCTATGGAGGA	TGG
CAN1.Z	GAAGCATATGTACAATGAGC	CGG
ADE2.Y	ACTTGAAGATTCTTTAGTGT	AGG
ADE2.Z	TTTTTCGATATCAAGAGGAT	TGG

Figure 1. Diagram of Cas9 complex and schematic of genetic constructs. (**A**) Illustration of Cas9 protein interacting with CRISPR gRNA to direct endonuclease activity proximal to the PAM sequence. (**B**) Design of the Cas9 and gRNA constructs. Cas9 gene contained a SV40 nuclear localization signal and was expressed under the Gal-L inducible promoter in CAN1 experiments and the TEF1 constitutive promoter in ADE2 experiments. The gRNA was expressed under the snoRNA SNR52 promoter and contained a terminator from the 3′ region of the yeast SUP4 gene. CAN1.Y and CAN1.Z were targeted to different loci in the CAN1 gene, whereas ADE2.Y and ADE2.Z were targeted to different loci in the ADE2 gene.

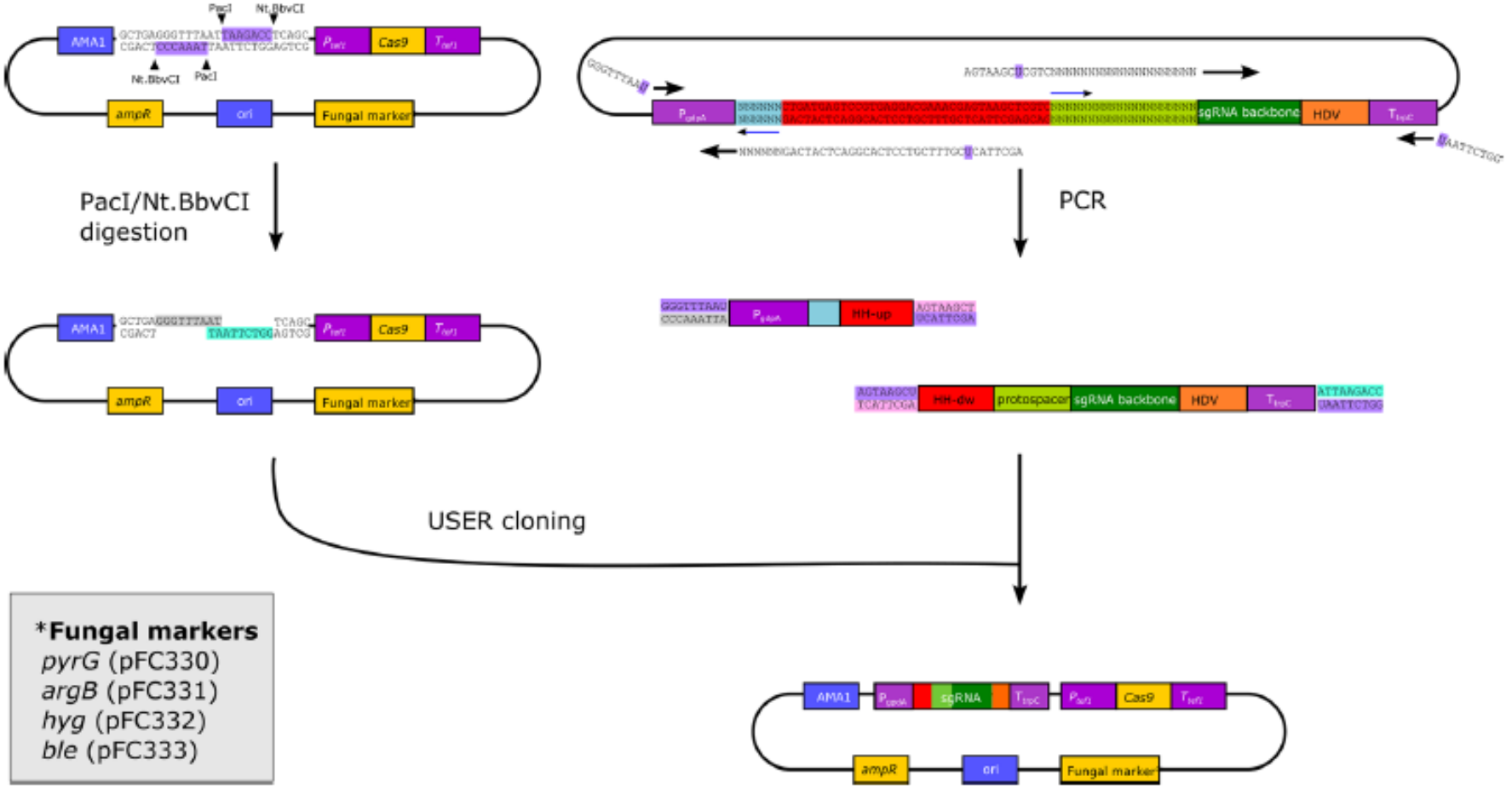


Fig 2. Construction of new CRISPR-Cas9 vectors for directed mutagenesis of filamentous fungi. Construction of fungal CRISPR-Cas9 vectors with variable sgRNA genes controlled by *gpdA* promoter and *trpC* terminator (no DNA elements are drawn to scale). The vector backbone for construction of new Fungal vectors for Cas9 induced genetic engineering are derived from the plasmid series pFC330-333. Sticky ends for USER cloning are achieved by opening the PacI/Nt.BbvCI USER cassette of pFC330-333 by the concerted action of restriction enzymes PacI and Nt.BbvCI (left side of panel). The two PCR fragments necessary for construction of the sgRNA gene, are both obtained by using pFC334 as template (right side of panel). This vector contains a protospacer for targeting yA (in light green), which is represented by 20 Ns indicating that it is not intended to match the primer; and in principle could be any sequence. The sections of the sgRNA gene encoding the variable parts of the transcript, the 20 bases of the protospacer (in light green) and the reverse complementing 6 bases of HH (in light blue), are introduced via tails added to the ends of the two primers that define the down- and upstream ends of the two PCR fragments, respectively. The position of the resulting inverted repeat located in the variable regions is indicated by blue arrows (top of panel). After amplification, the two PCR fragments are fused and inserted into vector pFC330-333 (Four variants exist) by USER cloning in a single step. For this purpose, each PCR fragment is generated by primers containing a tail with a uracil base (in purple). Elimination of the uracil bases in the PCR fragments by Uracil DNA glycosylase and DNA glycosylase-lyase Endonuclease VIII (USER Enzyme) results in the production of pairwise complementary overhangs at the ends of all fragments allowing selected ends to be fused in a directional manner. For simplicity, all complementary ends are visualized in the same color.

doi:10.1371/journal.pone.0133085.g002

Table 1. Mutation spectrum of RNA guided Cas9 mutagenesis.

Species	Target Gene	Colony #	Mutation	Wild-Type Target Sequence[1]	Mutated Target Sequence [2]
A. nidulans	*yA*	1, 3, 4, 8, 9, 10	1 bp deletion	GGCGGAGTATCATAACATCG	GGCGGAGTATCATAAC-TCG
	yA	5, 6	2 bp deletion	GGCGGAGTATCATAACATCG	GGCGGAGTATCATAA—TCG
	yA	7	1 bp deletion, 60 bp insertion	GGCGGAGTATCATAACATCG	GGCGGAGTATCATAAC-60 bp[4]-TCG
	yA	2	84bp insert	GGCGGAGTATCATAACATCG	GGCGGAGTATCATAACA-84 bp[3]-TCG
A. aculeatus	*albA*	1	1 bp deletion	CGGTTCTTCAACATGTCGCC	CG-TTCTTCAACATGTCGCC
	albA	2	10 bp deletion	CGGTTCTTCAACATGTCGCC	—-TTCTTCAACATGTCGCC
	pyrG	1	1 bp deletion	CCCACATCATCAACTGCAGCATC	ACA-CATCAACTGCAGCATC
	pyrG	2	2 bp deletion	CCCACATCATCAACTGCAGCATC	ACA—ATCAACTGCAGCATC
A. niger	*albA*	1	83 bp deletion	AGTGGGATCTCAAGAACTAC	50—Protospacer—13
	albA	2	83 bp deletion	AGTGGGATCTCAAGAACTAC	50—Protospacer—13
A.. carbonarius	*albA*	1	7 bp deletion	AGTGGGATCTCAAGAACTACTGG	AGTGGGATCT———-TACTGG
	albA	2	24 bp deletion	AGTGGGATCTCAAGAACTACTGG	AGTGGGATCT-24bp
A. luchuensis	*albA*	1	70 bp deletion	AGTGGGATCTCAAGAACTACTGG	AGTGGGATCTCAAGAAC—70-
A. brasiliensis	*albA*	1	11 bp deletion	AGTGGGATCTCAAGAACTACTGG	AGTGGGATCT————-GG
	albA	2	25 bp deletion, 1 bp insertion	AGTGGGATCTCAAGAACTACTGGATCCCCTAT	AGTG——C ————————-AT

[1]Underlined bp shows expected location for Cas9 induced DSBs.

[2]Hyphens indicate deleted bp

[3]GCCATTGTTGGCTCGTGAAGTGTACGGATTGATGTATCGTCGTATCTGCATATTGCCCCTGAGACTGATGATCATGTCTGTCGGA; match AN11611

[4]TTCAAAATCTCGGAGGCTGATTGTTCCACGATGCGGGTGACGGCTCCTCGGGGCGTTTCT; match AN10634

doi:10.1371/journal.pone.0133085.t001

Agaricus bisporus genome editing

- First CRISPR/Cas edited organism to receive green light from US Government
- targeting the family of genes that encodes polyphenol oxidase (PPO) – enzyme activity reduced by 30 %
- Resistant to browning
- “APHIS does not consider CRISPR/Cas9-edited white button mushrooms as described in your October 30, 2015 letter to be regulated”

Engineering of cyanobacteria and algae

- Engineering of photosynthetic organisms is promising area of modern biotechnology
- Nuclear or chloroplast transformation can be selected
- Most prominent targets of the engineering projects are biofuels and hydrogen production, followed by fine chemicals
- Chlamydomonas and Synechocystis are the most abundantly engineered organisms

Box 1. Nuclear versus chloroplastic transformation

Integration of transgenes into the chloroplast has important advantages. It enables controlled site-directed recombination of constructs and results in high expression levels with no silencing drawbacks (Table I). However, nuclear transformation might enable a wider range of possibilities both for transgenic protein expression (e.g. excretion, different cell-compartment expression, and glycosylation) and for manipulation of algal metabolism (gene inactivation or overexpression, and gain of additional pathways) (Table I).

Table I. Main characteristics of nuclear and chloroplastic transformations

	Nuclear	Chloroplastic
Cell compartment of expression	Extracellular, cytosol and chloroplast, among others	Chloroplast
Recombination machinery for integration of exogenous DNA	Mostly non-homologous	Homologous
Gene silencing	Probable	Not probable
Inheritance of integrated gene	Mendelian	Maternal
Level of expression (gene copy number)	Low to intermediate	High
Co-transformation of different markers	High	High
Versatility to express genes from different organisms	Intermediate to low	High
Glycosylation pattern of proteins	Similar to plants and animals	None

Box 2. Main problems associated with foreign gene expression in microalgae

- Inadequate method of DNA delivery
- No integration into the chromosome
- Inadequate recognition of the promoter region
- Biased codon usage
- Lack of adequate regulatory sequences
- Incorrect polyadenylation
- Inappropriate nuclear transport
- Instability of mRNA
- Positional effects
- Silencing by methylation
  Epigenetic silencing mechanisms

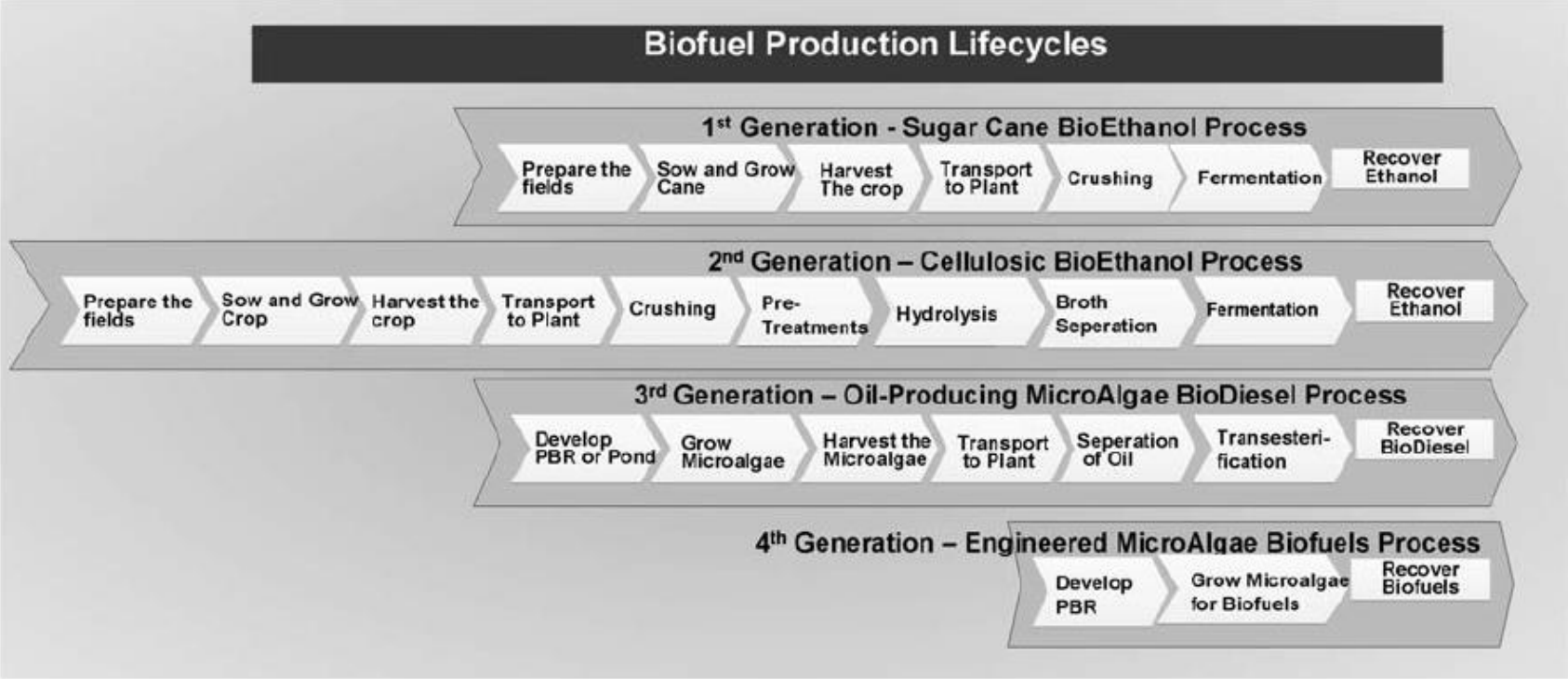


Fig. 5 Comparison of the typical bioprocess steps required for four generations of biofuels production.

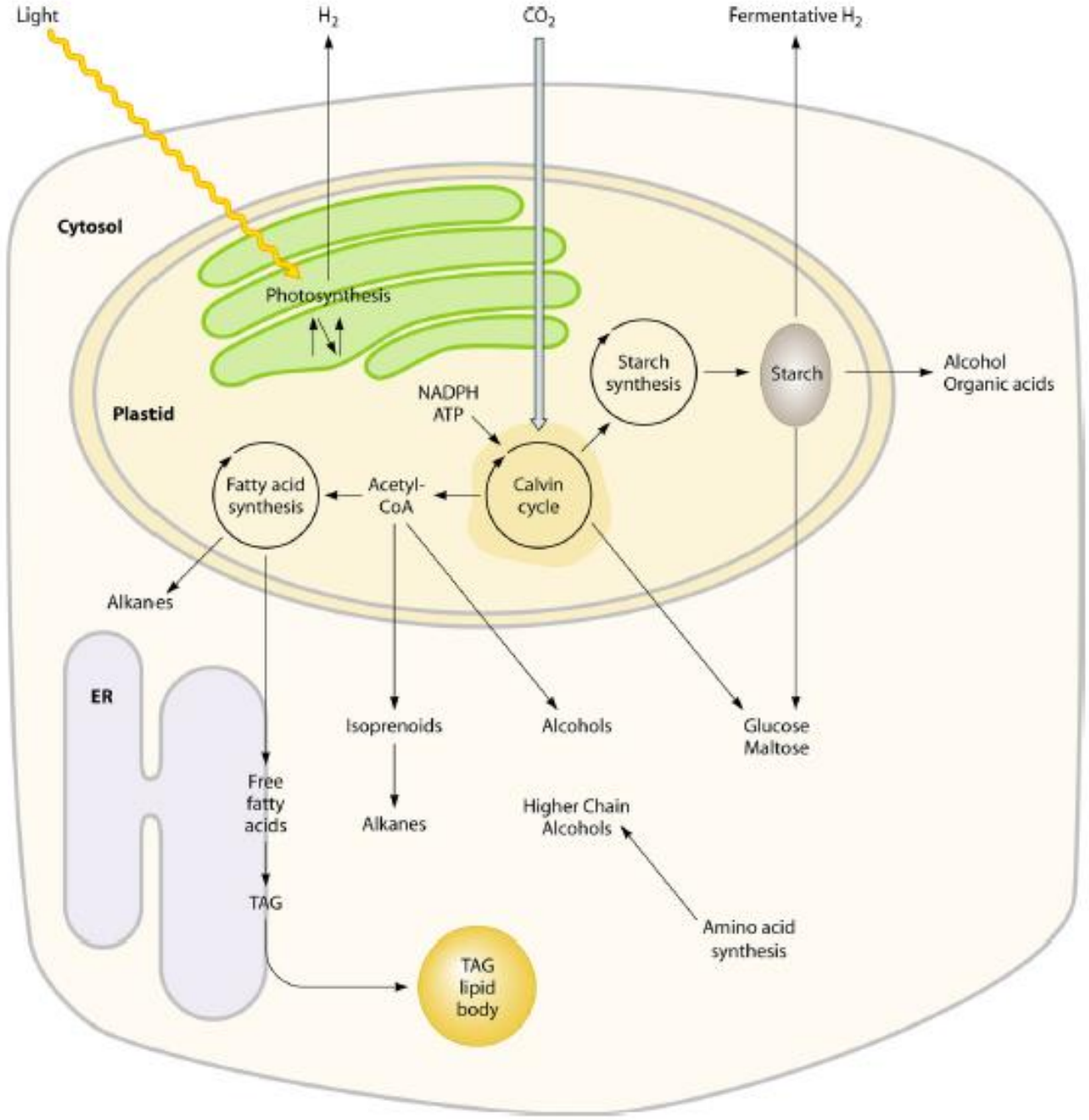


FIG. 1. Microalgal metabolic pathways that can be leveraged for biofuel production. ER, endoplasmic reticulum.

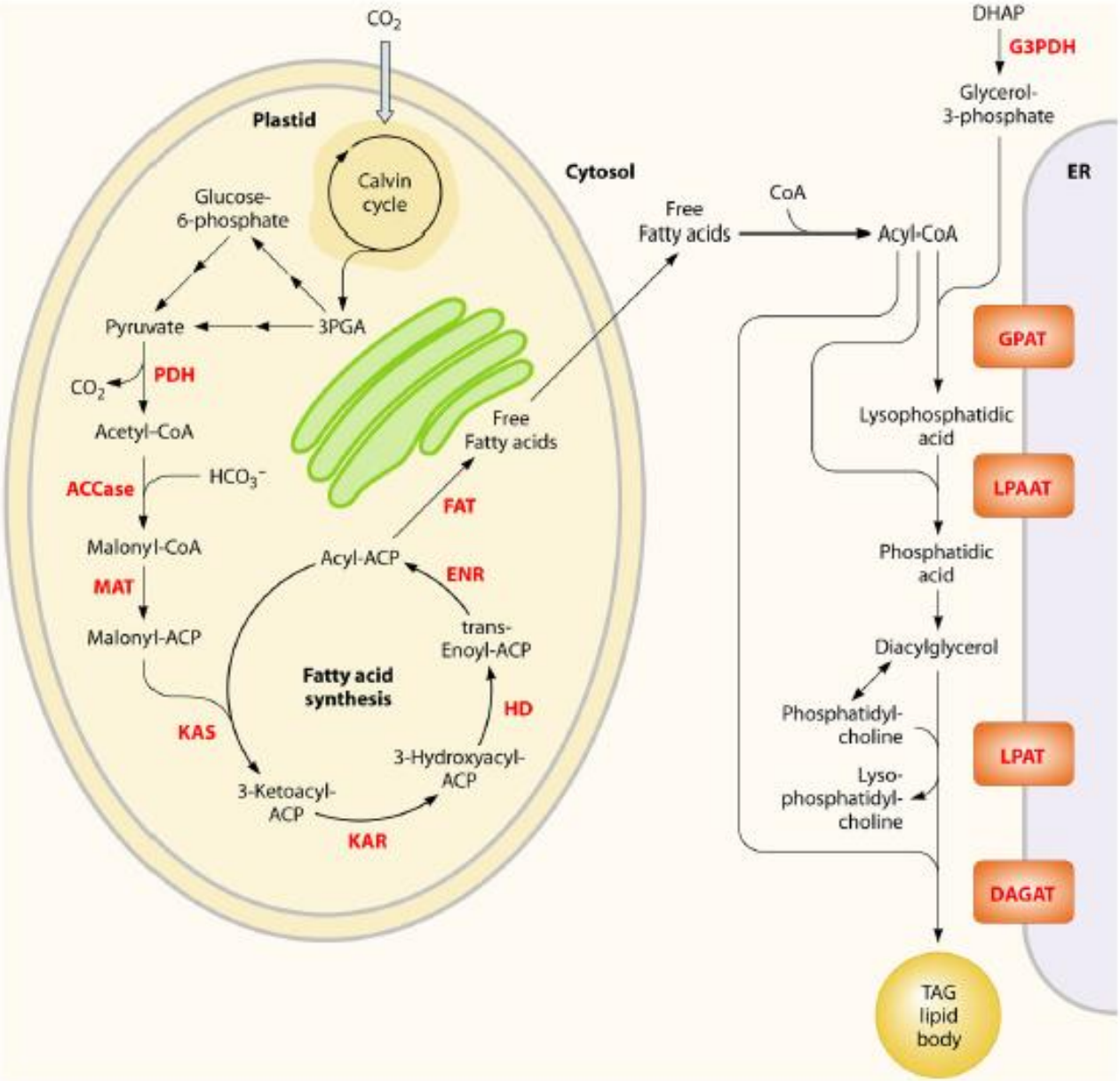


FIG. 2. Simplified overview of the metabolites and representative pathways in microalgal lipid biosynthesis shown in black and enzymes shown in red. Free fatty acids are synthesized in the chloroplast, while TAGs may be assembled at the ER. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; DAGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; ENR, enoyl-ACP reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, gycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl-ACP dehydratase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LPAAT, lyso-phosphatidic acid acyltransferase; LPAT, lyso-phosphatidylcholine acyltransferase; MAT, malonyl-CoA:ACP transacylase; PDH, pyruvate dehydrogenase complex; TAG, triacylglycerols.

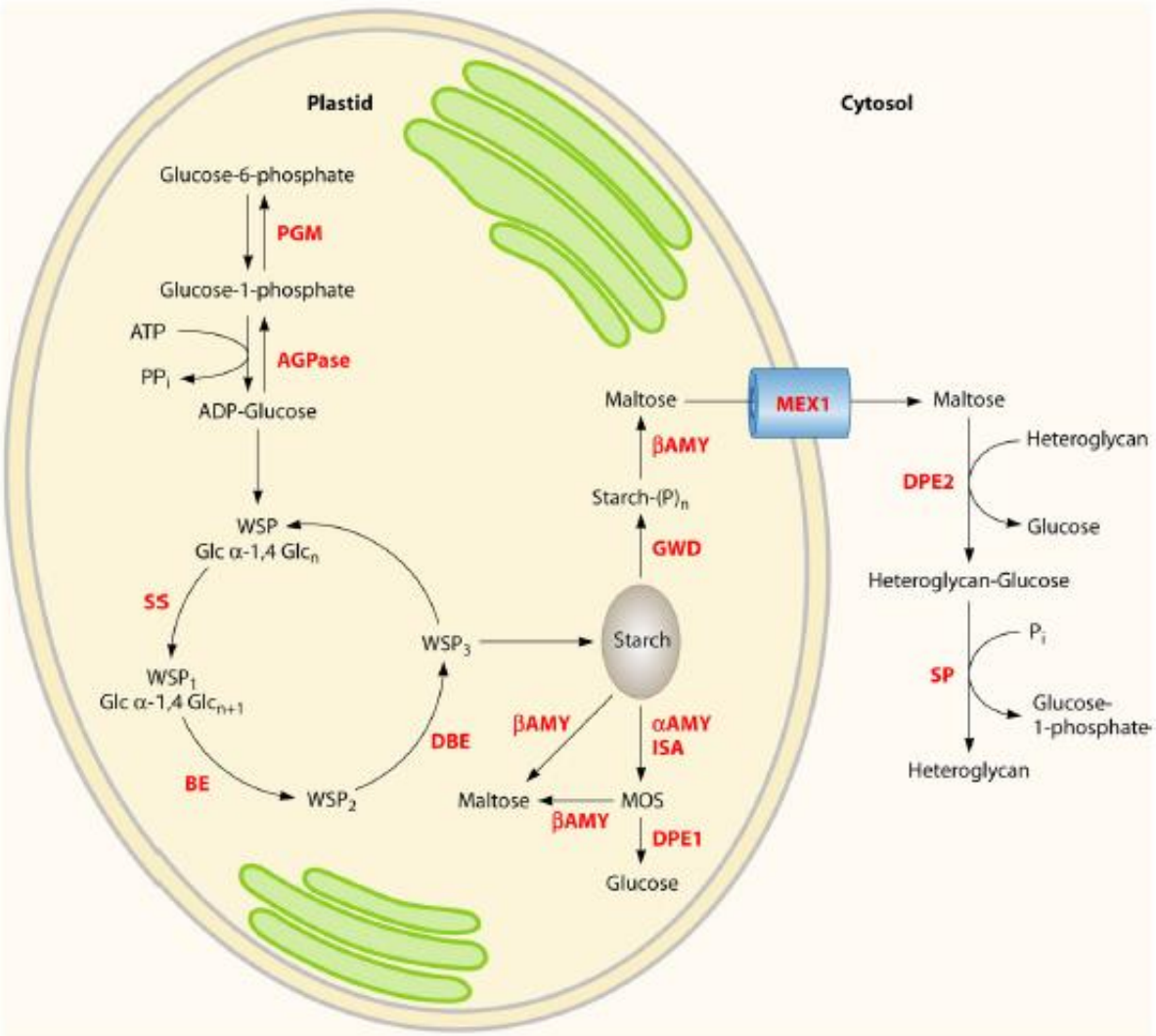


FIG. 3. Starch metabolism in green microalgae. The metabolites and simplified representative pathways in microalgal starch metabolism are shown in black, and enzymes are shown in red. Glucans are added to the water soluble polysaccharide (WSP) by α-1,4 glycosidic linkages (WSP_1) until a branching enzyme highly branches the ends (WSP_2). Some of these branches are trimmed (WSP_3), and this process is repeated until a starch granule is formed. Phosphorolytic [Starch-$(P)_n$] and hydrolytic degradation pathways are shown. αAMY, α-amylase; AGPase, ADP-glucose pyrophosphorylase; βAMY, β-amylases; BE, branching enzymes; DBE, debranching enzymes; DPE, disproportionating enzyme (1 and 2) α-1,4 glucanotransferase; Glc, glucose; GWD, glucan-water dikinases; ISA, isoamylases; MEX1, maltose transporter; MOS, malto-oligosaccharides; PGM, plastidial phosphoglucomutase; P, phosphate; P_i, inorganic phosphate; PP_i, pyrophosphate; SP, starch phosphorylases; SS, starch synthases.

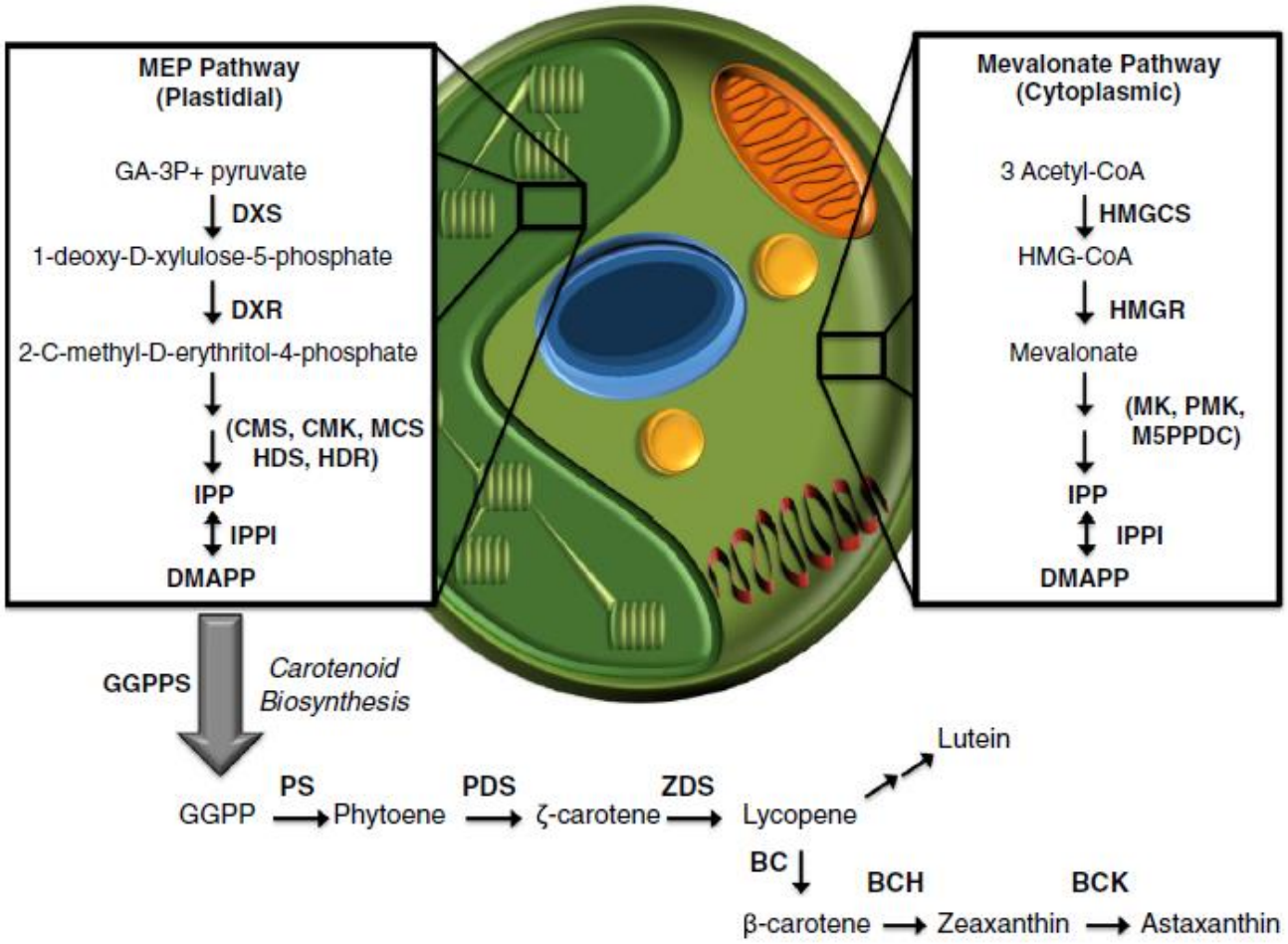


Fig. 2 Algal terpenoid biosynthesis pathways. Not all intermediates are displayed. Plastidial carotenoid biosynthesis represents one potential fate of DMAPP. Plastidial MEP components: *GA-3P* glyceraldehyde 3-phosphate; *DXS* 1-deoxy-D-xylulose 5-phosphate (DOXP) synthase; *DXR* DOXP reductase; *CMS* 2-c-methyl-D-erythritol 2-phosphate synthase; *CMK* 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; *MCS* methyl-erythritol-cyclo-diphosphate-synthase; *HDS* hydroxy-methyl-butenyl-diphosphate (HMBPP) synthase; *HDR* HMBPP reductase; *IPP* isopentyl diphosphate; *DMAPP* dimethylallyl diphosphate. Cytoplasmic mevalonate components: *HMGCS* 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; *HMGR* HMG-CoA reductase; *MK* mevalonate kinase; *PMK* phosphomevalonate kinase; *M5PPDC* mevalonate-5-diphosphate decarboxylase; *IPPI* isopentyl diphosphate isomerase. Carotenoid biosynthetic components: *GGPPS* Geranylgeranyl diphosphate (GGPP) synthase; *PS* phytoene synthase; *PDS* phytoene desaturase; *ZDS* zeta-carotene desaturase; *BC* beta-cyclase; *BCH* beta-carotene hydroxylase; *BCK* beta-carotene ketolase

Table 1 Summary of the genetically engineered strains with improved H_2 production

Barriers	Target	Strain	Genetic engineering technique	Phenotype	H_2 production vs control strain	Ref
O_2 sensitivity	D1 protein	*L1591-N230Y*	Site-directed mutagenesis	↑quantum yield of photosynthesis ↑ respiration rate	~20 fold↑ compared to WT 11/32b	Torzillo 2009
	PSII subunit O	*antiPSBO*	RNA interference	↑hydrogenase ↓ Fv/Fm	~10 fold↑ compared to WT *Chlorella sp. DT*	Hsin Di Lin 2013
	PSII	*cyc6nac2.49*	Random mutagenesis	↓ O_2	~3 fold↑ compared to *nac2-26*	Surzycki 2007
	P/R ratio	*apr1+* glycoaldehyde	DNA insertional mutagenesis	↑respiration rate ↓photosynthesis rate	~2-3 fold↑ compared to CC-425	Ruhle 2008
	Leg hemoglobin	CC-849 *+codon optimized hemH-lbA*	Heterologous expression from Bradyrhizobium and Glycine max in *Chlamydomonas*	↓growth ↑O_2 consumption	~4-fold ↑ compared to CC-849 *+non codon optimized hemH-lbA*	Wu 2010 Wu 2011
	Pyruvate oxidase	*ccHPC*	Heterologous expression from *E.coli* in *Chlamydomonas*	↓O_2 evolution	~3 fold ↑ compared to CC-503	Xu 2011
	Sulfate permase	*antisulp*	RNA Antisense	↓uptake sulfate ↓O_2 evolution ↓steady state levels of PSII	~4 fold ↑ compared to CW-15	Chen 2005

Proton gradient	Cyclic Electron Flow PGRL1 protein	*pgrl1*	DNA insertional mutagenesis	↑ETR ↓NPQ	~3 fold ↑ compared to 137C	Tolleter 2011
State transition	State transitions	*stm6*	DNA insertional mutagenesis	↑ starch reserves ↑ respiration No state transitions ↓ PSII activity	~5-13 fold↑ compared to CC-1618	Kruse 2005, Volgusheva 2013
Photosynthetic efficiency	Antenna size	*tla1*	DNA insertional mutagenesis	↓ Chl/reaction center ↑O_2 evolution/Chl ↑ H_2 rate/Chl	~4 fold ↑ compared to CC-4169	Kosourov 2011, Polle 2003
	Light harvesting complex LHCBM1,2,3	*stm6GLC4lo1*	RNA interference	↓Chl ↑ H_2 rate/Chl	~2 fold ↑ compared to *stm6GLC4*	Oey 2013
Competition for electron	Rubisco large subunit	CC-2803	DNA insertional Mutagenesis	Light sensitive Reduced RuBisCo Activity ↓O_2 evolution	~2 fold ↑ compared to 137C	Heimscheier 2008
	Rubisco small subunit	*Y67A*	Site-directed mutagenesis	Reduced RuBisCo Activity ↓O_2 evolution	~10 fold↑ compared to *RBCS-T60-3*	Pinto 2013
Low reductant flux	Sugar reserve	*stm6GLC4*	Heterologous expression of HUP1 from Chlorella in *Chlamydomonas*	Import glucose ↑ growth rate	~1.5 fold↑ compared to *stm6*	Doebbe et al. 2007, 2010
	Starch enzyme	*std3 sda6*	DNA insertional mutagenesis	↑residual starch amounts	~1.5 and 1.2 fold↑ compared to 137C	Chochois 2010
Low level of hydrogenase	Hydrogenase from *Chlorella sp.DT*	*C.s DT hydA*	Homologous overexpression in Chlorella	↑Fv/Fm	~7 fold↑ compared to WT *Chlorella sp. DT*	Chien 2012

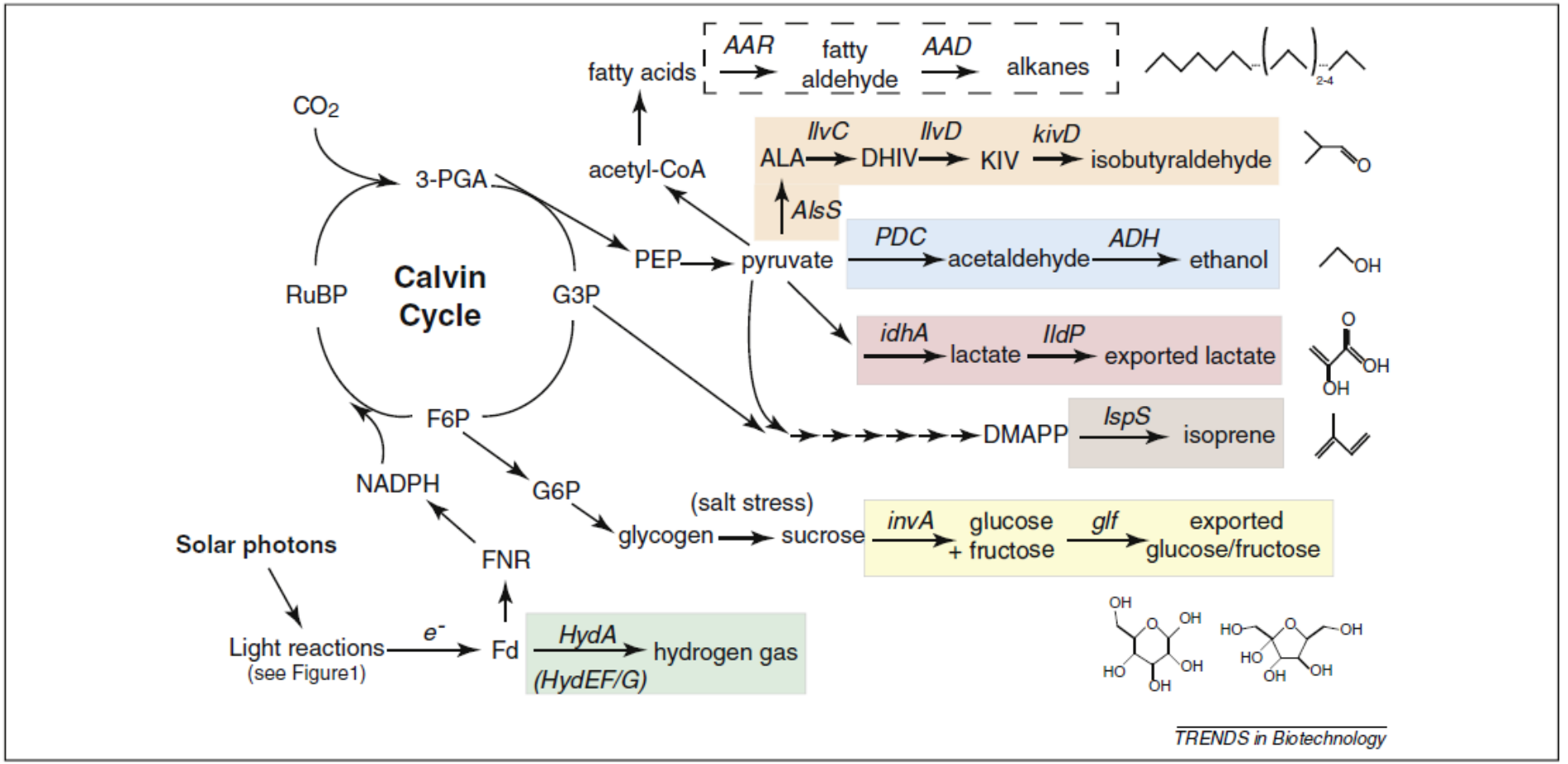
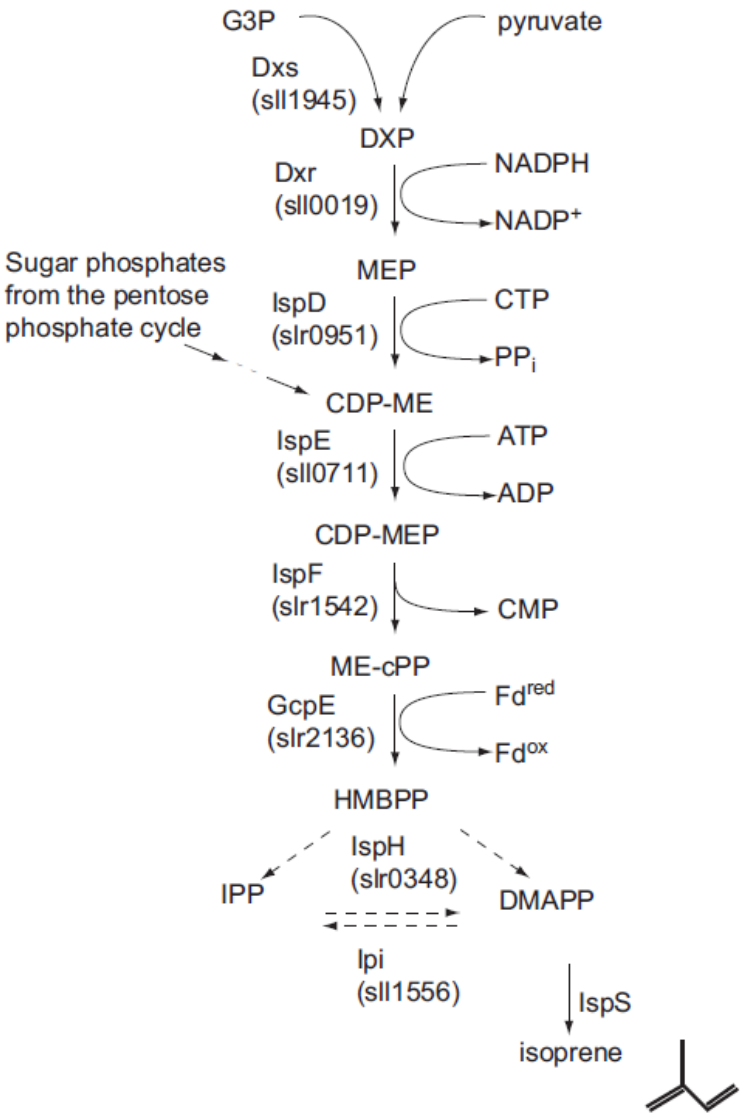


Figure 2. Schematic representation of engineered biochemical pathways in cyanobacteria. Core metabolism of photosynthetic processes is shown in black text. Branch points utilized for the production of various compounds discussed in this review are indicated (highlighted pathways) with relevant enzymes catalyzing specific reactions indicated in italics. Abbreviations: 3-PGA, 3-phosphoglycerate; AAD, aldehyde decarbonylase; ADH, alcohol dehydrogenase II; ALA, 2-acetolactate; AlsS, acetolactate synthase; DHIV, 2,3-dihydroxy-isovalerate; F6P, fructose 6-phosphate; FNR, ferredoxin NADP+ reductase; G6P, glucose 6-phosphate; HydA, [FeFe] hydrogenase; HydEF/G, hydrogenase maturation factors; IdhA, lactate dehydrogenase; IlvD, dihydroxy-acid dehydratase; IlvC, acetohydroxy acid isomeroreductase; PDC, pyruvate decarboxylase; PEP, phosphoenolpyruvate.



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Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism

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Fig. 1. The MEP pathway for isoprene biosynthesis. Abbreviations used: G3P=glyceraldehyde 3-phosphate; DXP=deoxyxylulose 5-phosphate; MEP=methylerythritol 4-phosphate; CDP-ME=diphosphocytidylyl methylerythritol; CDP-MEP=CDP-ME 2-phosphate; ME-cPP=methylerythritol 2,4-cyclodiphosphate; HMBPP=hydroxymethylbutenyl diphosphate; IPP=isopentenyl diphosphate; DMAPP=dimethylallyl diphosphate. Enzymes: Dxs=DXP synthase; Dxr=DXP reductoisomerase; IspD=CDP-ME synthase; IspE=CDP-ME kinase; IspF=ME-cPP synthase; GcpE (IspG) HMBPP synthase; Fd=ferredoxin; IspH=HMBPP reductase; Ipi=IPP isomerase; IspS=isoprene synthase. Where applicable, corresponding ORF names in the *Synechocystis* genome database (http://genome.kazusa.or.jp/cyanobase/ (Kaneko and Tabata, 1997)) are given in parentheses. In addition to reactants G3P and pyruvate, the MEP pathway consumes reducing equivalents and cellular energy in the form of NADPH, reduced ferredoxin, CTP and ATP, ultimately derived from photosynthesis (see also Ershov et al., 2002; Sharkey et al., 2008).

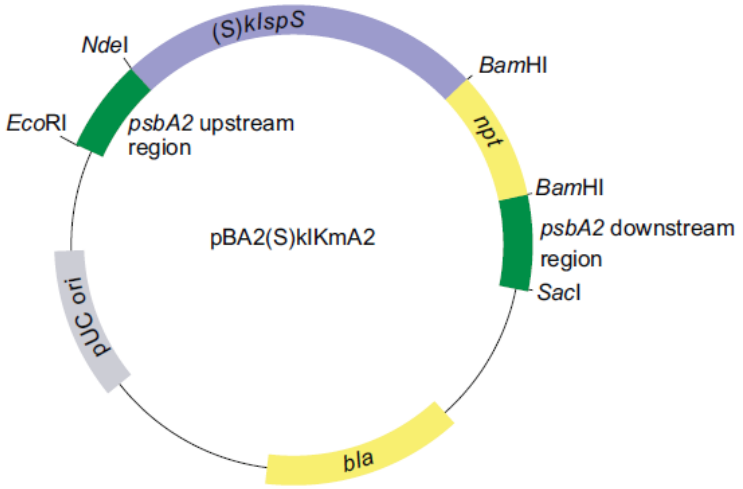


Fig. 4. Plasmid constructs for the transformation of *Synechocystis*. The two versions of the isoprene synthase gene, *klspS* and *SklspS* (indicated in the figure as (*S*)*klspS*), were each cloned in a pBluescript-based plasmid, also containing an antibiotic resistance cassette and the flanking sequences of *psbA2*. The resulting plasmids were used for insertion into the *Synechocystis* genome via double homologous recombination. Restriction sites used for cloning are indicated (see Section 2). *npt*=neomycin phosphotransferase gene, conferring kanamycin resistance; *bla*=β-lactamase gene, conferring ampicillin resistance.

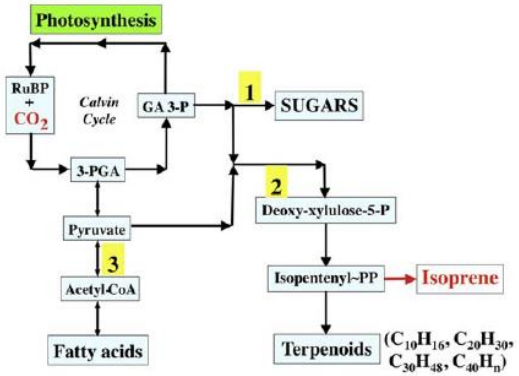
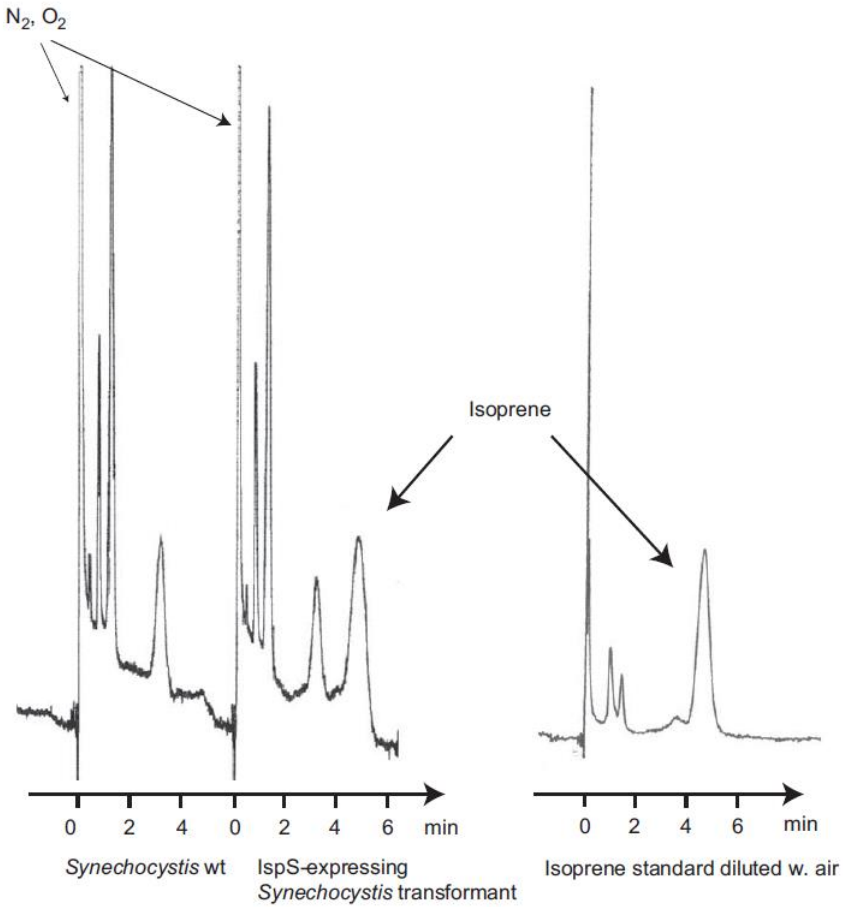


Fig. 9. Photosynthetic carbon flux and partitioning among the sugar (1), terpenoid (2) and fatty acid (3) biosynthetic pathways. Carbon allocation is primarily directed toward sugar biosynthesis (80–85%), with fatty acid biosynthesis (~10%) and terpenoid biosynthesis (3–5%) lagging far behind that of sugar.



Trait	Trait description	Gene constructs
3	TE with high activity for 16:0 and 18:0 FA-ACPs	*FatB* from *Arabidopsis thalaiana* (GenBank: NP_172327)
16	Inactivation of AAS activity	Insertional inactivation of *S.* 6803 *AAS* gene (locus *slr1609*)
30	Enhanced activity of FAR andFAD	Operon with *S.* 6803 *FAR* and *FAD* genes (loci *sll0208* and *sll0209*, respectively)

AAS: Acyl-ACP synthetase
ACC: Acetyl-CoA carboxylase
ACP: Acyl carrier protein
AGPAT: Acylglycerol-3-phosphate acyltransferase
CBB cycle: Calvin-Benson-Bassham cycle
DHAP: Dihydroxy-acetone phosphate
FA acyl-ACP: Fatty acyl-ACP
FAD: Fatty aldehyde decarbonylase
FAR: Fatty acyl-ACP reductase
FDH: Formate dehydrogenase
FFA: Free fatty acids
GPAT: Glycerol-3-phosphate acyltransferase
PHAS: Polyhydroxyalkanoate synthase
PHB: Polyhydroxybutyrate
TE: Thioesterase

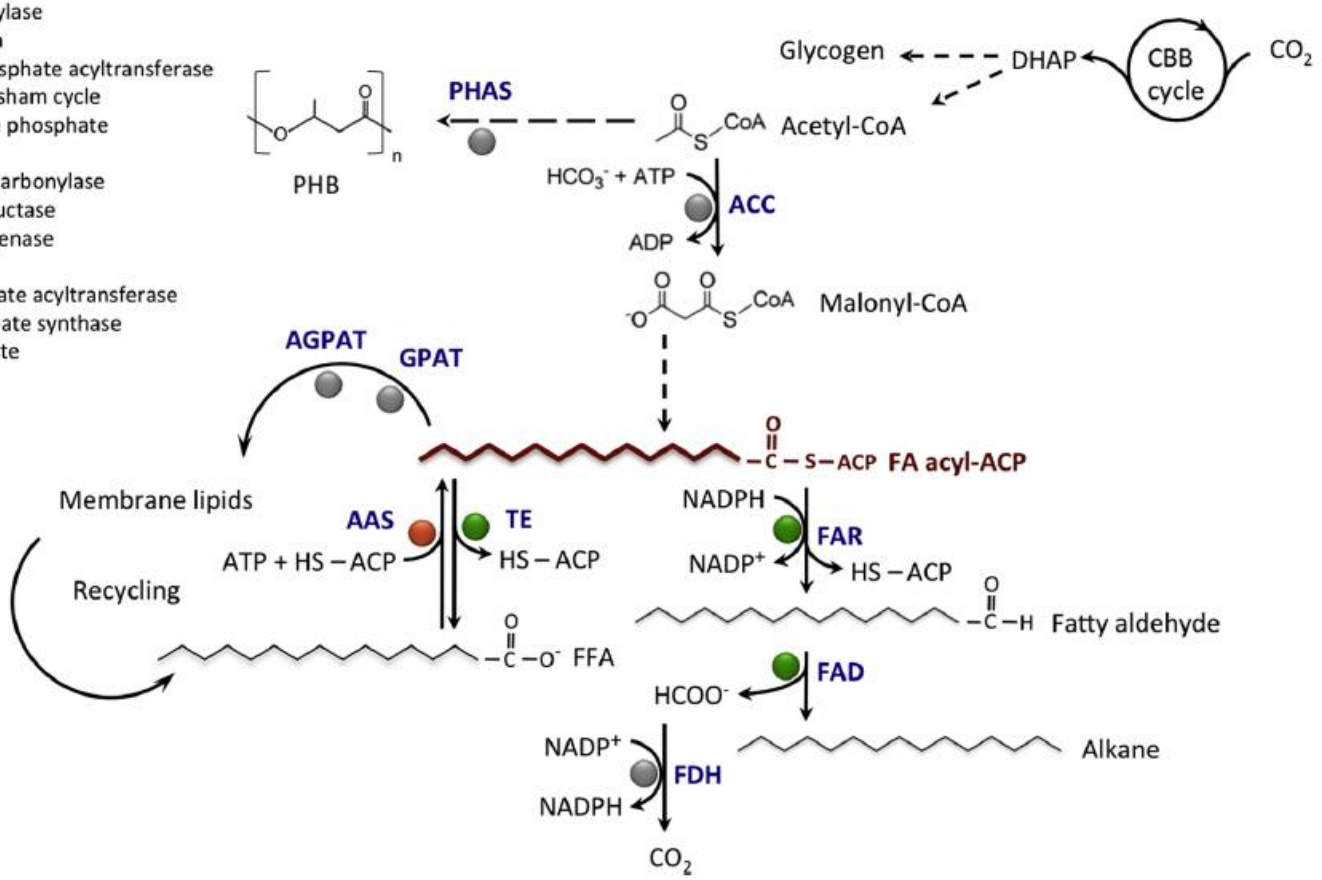


Fig. 1. Lipid metabolism in *S.* 6803-FUEL strains. Key enzymes affecting FA and alkane biosynthesis are shown (spheres). Enzyme activities targeted in this study are indicated with green for introduced/enhanced activities and red for blocked activity. Traits used to designate the strains are shown. The crossroad position of FA acyl-ACP in FA and alkane biosynthesis is indicated.