Electronic Supplementary Material (ESI) for Green Chemistry.

Supporting information

Multi-level engineering of *Saccharomyces cerevisiae* for synthesis and accumulation of retinal

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1 Experimental Procedures

1.1 Strains, culture media and reagents

Escherichia coli strain DH5α was used for plasmid construction purposes, and the strains harboring plasmid were cultivated at 37ºC in Luria-Bertani broth (10.0 g/L tryptone, 5.0 g/L yeast extract, and 10.0 g/L NaCl) with 100 μg/mL ampicillin. *S. cerevisiae* BY4741 (MATa, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*) was used as the parental strain for all subsequent strain constructions. *S. cerevisiae* BY4741 was cultured in rich YPD medium (10 g/L yeast extract, 20 g/L tryptone and 20 g/L glucose), and the engineered strains with different auxotrophic selection markers were grown in synthetic complete (SC) medium with appropriate dropouts. All restriction enzymes, T4 ligase, Taq polymerase and High-fidelity Phusion polymerase were obtained from New England Biolabs (Beverly, MA, USA). Gel extraction kit and plasmid purification kit were purchased from BioFlex (Shanghai, China). Ampicillin and 5-fluoroorotic acid were purchased from Sangon Biotech (Shanghai, China). Phleomycin was purchased from (Invivo, China). The standard retinal (Cat. No. A122355), retinol (Cat. No. V111674), and *β*-carotene (Cat. No. C110502) were purchased from Aladdin Biotech (Shanghai, China). Lycopene (Cat. No. 013226884) was purchased from Adamas Biotech (Shanghai, China).

1.2 Plasmid construction

Oligonucleotides used for plasmid construction were synthesized by Sangon Biotech (Shanghai, China) and the details are listed in Supplementary Table S1. Plasmid YEplac195-YB/I/E was a kind gift from Prof. Gerhard Sandmann. The *BLH* gene from the marine bacterium 66A03 was kindly provided by Prof. Lidan Ye from Zhejiang University. Plasmid pδBLE2.0-ERG13/ERG10, pδBLE2.0-ERG12/tHMG1, pδBLE2.0-ERG19/ERG8 and pδBLE2.0-IDI1/ERG20 from our previous report[17] was used for optimizing the MVA pathway. Plasmid pRS426-ACL was derived from pESC-URA with insertion of *ACL1* and *ACL2* gene from *Y. lipolytica*. For expressing *crtE*, *crtYB* and *crtI* genes under the galactose-regulated promoter, all the genes were PCR amplified from the plasmid YEplac195-YB/I/E, and subsequently inserted into pδBLE1.1[1] to yield pδBLE -CrtE, pδBLE-CrtYB and pδBLE-CrtI. The *BLH* gene was inserted into pRS425GAL1 to yield pRS425GAL1-BLH. The guide RNA (gRNA) expressing plasmids were constructed via the golden-gate assembly method. All plasmids used in this study are listed in the Supplementary Table S2.

1.3 Genetic modification of budding yeast

For CRISPR/Cas9 mediated genome editing, the standard protocol of *S. cerevisiae* transformation was carried out by electroporation with minimal modification[2]. 50 μl of yeast cells together with approximately 2 μg mixture of genome editing cassette was electroporated in a 0.2 cm cuvette at 1.6 kV. After electroporation, cells were immediately mixed with 900 μl YPD medium and recovered a rotary shaker for 1 h. Following that, cells were collected by centrifugation at 3000 rpm for 5 min, washed and resuspended in 50 μl ddH_2O. Next, 50 μl cells were plated on SC plate with appropriate dropouts. Colonies were randomly picked from the plate and subjected to diagnostic PCR verification of genome editing events. Successful genome manipulations were confirmed by diagnostic PCR before proceeding to the next round of genetic modifications. Subsequently, gRNA expressing plasmid was eliminated via counter-selection with 1 g/l 5-fluoorotic acid (5-FOA), and the Cas9-expressing plasmid was removed via a series dilution and confirmed by diagnostic PCR.

For combinatorial optimization of the MVA pathway, plasmid pδBLE2.0-ERG13/ERG10, pδBLE2.0-ERG12/tHMG1, pδBLE2.0-ERG19/ERG8, and pδBLE2.0-IDI1/ERG20 were served as template and primer pair F_Delta_Int/R_Delta_Int was used for the subsequent PCR amplification of the linear genome integration cassettes as previously described[1]. Cells were directly spotted on YPD agar plate supplemented with 240 μg/mL phleomycin, to achieve combinatorial integration of the MVA modules into yeast chromosomes.

1.4 *De novo* synthesis of carotenoids and retinoids in the engineered yeast cells

For small-scale characterization of carotenoid production in the engineered yeasts, experiments were carried out in 14 ml tubes supplemented with 2 ml culture medium. The strains were picked from the plate and inoculated

into SC medium with appropriate dropouts. Next day, 14 ml sterile tubes containing 2 ml medium supplemented with 2% glucose and 20 μM copper sulphate were inoculated with fresh overnight cultures to an initial OD_{600} of 0.1. After 48 h cultivation, 100 μL of cell culture was taken for determining *β*-carotene using high-performance liquid chromatography (HPLC).

For the retinoid production, experiments were carried out in shake-flasks. 100 ml flasks containing 20 ml medium supplemented with 2% glucose and 20 μM copper sulphate were inoculated with fresh overnight cultures to an initial OD_{600} of 0.1. Subsequently, 4 ml dodecane was added for *in situ* extraction of retinoids during the fermentation process. The OD_{600} and retinoid levels were continuously monitored for 120 h. The organic phase was used for determining the retinoid levels using the HPLC system.

1.5 Analysis of carotenoids and retinoids

In the two-phase culture system with a dodecane overlay, the upper dodecane phase containing the retinoids was collected and centrifuged for 10 min at 14,000 rpm to remove all cellular particles. The intracellular content of carotenoids was extracted from yeast cell pellets with acetone. Specifically, the harvested cells were resuspended with 1ml acetone in a 2 ml screwed cap tube and crushed by a bead ruptor (OMNI, USA). After centrifugation, supernatants were collected in a new brown tube for measuring the carotenoid levels. The acetone extracts and dodecane phases were analyzed with HPLC (LC-20A, Shimadzu) equipped with a C18 column (250 mm × 4.6 mm, 5 μm). The mobile phases were 95:5 and 50:50 methanol and acetonitrile for the retinoid and carotenoids analyses, respectively. A flow rate of 1.5 ml/min (retinoid) or 1.0 ml/min (lycopene and *β*-carotene) and column temperature of 40°C were applied for the HPLC analysis. The detection wavelengths were set as following: 370 nm (retinal), 340 nm (retinol), 450 nm (lycopene), and 450 nm (*β*-carotene). All standards (retinal, retinol, lycopene and *β*-carotene) were dissolved in methanol for the subsequent plotting of the standard curve.

2 Supporting Figures

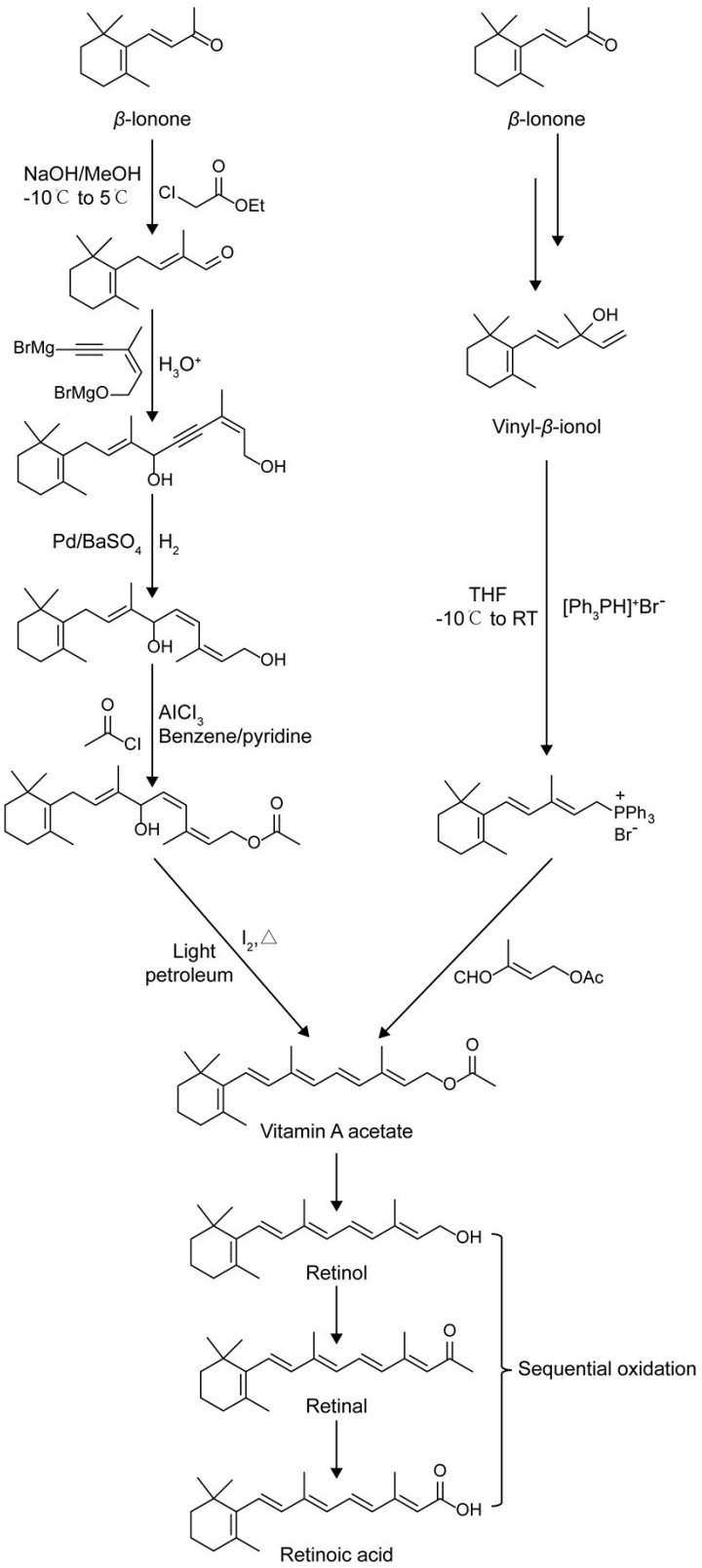

Figure S1. Roche (Left) and BASF (Right) synthesis of retinoids.

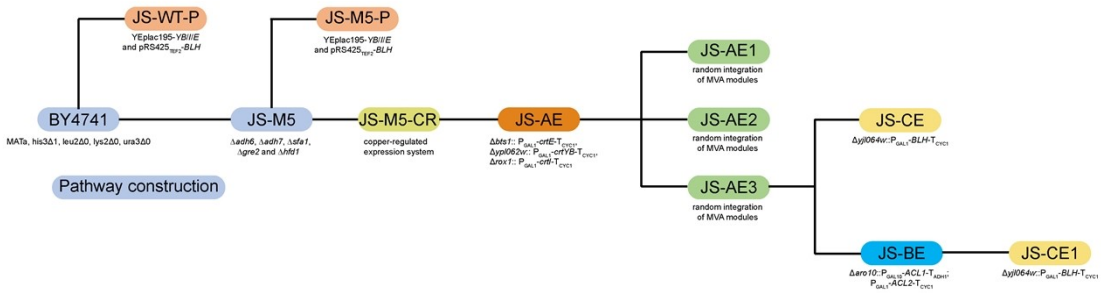

Figure S2. Flowchart of yeast strain construction in this study.

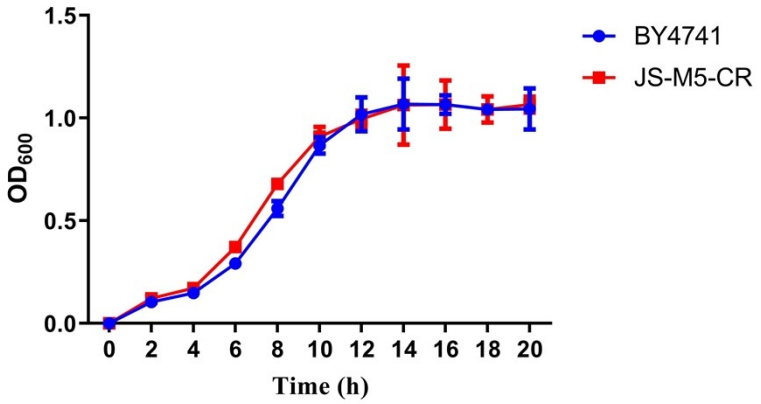

Figure S3. Evaluation the growth curves of strain BY4741 and JS-M5-CR. The growth data were collected by microplate reader.

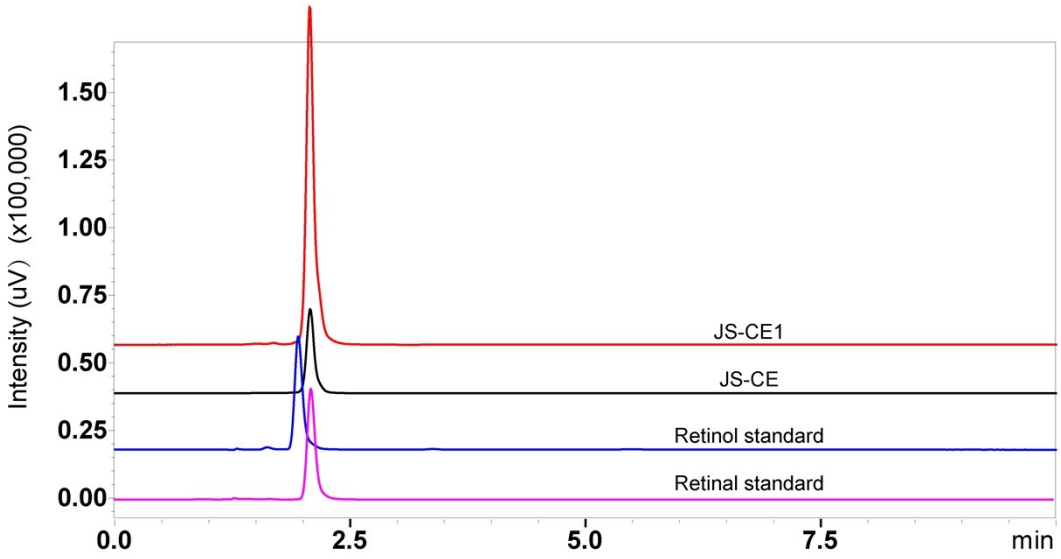

Figure S4. Representative HPLC of retinal production in strain JS-CE and JS-CE1 after 120 h cultivation.

3 Supporting Tables

Table S1. Oligonucleotides used in this study.

Name	Description
F_gRNA.adh6	TTGGTCTCAGATGCTAGGGCCCAAGTCAAACAGGTTTTAGAGCTAGAAATAG
F_gRNA.adh7	TTGGTCTCAGATGACCACCACACAATAATGGAGGTTTTAGAGCTAGAAATAG
F_gRNA.sfa1	TTGGTCTCAGATGCTTGGGATTAACAAAATCCGGTTTTAGAGCTAGAAATAG
F_gRNA.hfd1	TTGGTCTCAGATGGAAATGGCAATAACAAGTGGGTTTTAGAGCTAGAAATAG
F_gRNA.gre2	TTGGTCTCAGATGAAAGGCCGAGAATTTAACGGGTTTTAGAGCTAGAAATAG
F_gRNA.bts1	TTGGTCTCAGATGGATAATGCTCCCTTGAGAAGGTTTTAGAGCTAGAAATAGC
F_gRNA.ypl062w	TTGGTCTCAGATGGCCTTATTCCCGTGGGGATGGTTTTAGAGCTAGAAATAGC
F_gRNA.rox1	TTGGTCTCAGATGTGTCTCGTTGAAGGGTCGTGGTTTTAGAGCTAGAAATAGC
F_gRNA.yjl064w	TTGGTCTCAGATGGAATTCTGTAGCAAACGCTGGTTTTAGAGCTAGAAATAGC
F_gRNA.aro10	TTGGTCTCAGATGGTTTTGAGTATCAAGTTGAGGTTTTAGAGCTAGAAATAG
R_SUP4	TTGGTCTCAAAAGAGACATAAAAAACAAAAAAAG
F-adh6-Del	ATGTCTTATCCTGAGAAATTTGAAGGTATCGCTATTCAATTTACCTTAG
R-adh6-Del	CTAGTCTGAAAATTCTTTGTCGTAGCCGACTAAGGTAAATTGAATAGCG
F-adh7-Del	ATGCTTTACCCAGAAAAATTTCAGGGCATCGGTATTTCCTTTACTTTGG
R-adh7-Del	CTATTTATGGAATTTCTTATCATAATCGACCAAAGTAAAGGAAATACCG
F-sfa1-Del	ATGTCCGCCGCTACTGTTGGTAAACCTATTAAGTGCATTTGCTTAAGAA
R-sfa1-Del	CTATTTTATTTCATCAGACTTCAAGACGGTTCTTAAGCAAATGCACTTA
F-gre2-Del	ATGTCAGTTTTCGTTTCAGGTGCTAACGGGTTCATTGCCACTGCCTCCC
R-gre2-Del	TTATATTCTGCCCTCAAATTTTAAAATTTGGGAGGCAGTGGCAATGAAC
F-hfd1-Del	ATATTCTAAAACCATAGCCATAGTAATTTATCACCAACATGTCACACCCCGCGTTAAC
R-hfd1-Del	CTTATACATCAAATAATTAATTAACCTTAAACATTACGTTTAGTACAACGGTGACGCCG
F-bts1-Int	ATGGAGGCCAAGATAGATGAGCTGATCAATAATGATCCTGGAGCGACCTCATGCTATAC
R-bts1-Int	TCACAATTCGGATAAGTGGTCTATTATATATAACAATTCGCTTCGAGCGTCCCAAAACC
F-ypl062w-Int	ATGATAGAATTGGATTATGTAAAAGGTGAAGATACCATTGGAGCGACCTCATGCTATAC
R-ypl062w-Int	CTATATCGCATTCGTTGCACTCACCGTTCCCAAGAGGAGACTTCGAGCGTCCCAAAACC
F-rox1-Int	ATGAATCCTAAATCCTCTACACCTAAGATTCCAAGACCCAGAGCGACCTCATGCTATAC
R-rox1-Int	TCATTTCGGAGAAACTAGGCTAGTTTTAGCGGTGACCTCACTTCGAGCGTCCCAAAACC
F-yjl064w-Int	ATGACACTTGTAGTATATCTAACTCGGTTTTCTTCCACTAGAGCGACCTCATGCTATAC
R-yjl064w-Int	TCAGGCTAACACAATGAACAACGAGACTAGTGGTAAAGAACTTCGAGCGTCCCAAAACC
F-aro10-Int	GTTTATTAATACTACATAAAATCTGATATAAAACATATTTTCTTCGAGCGTCCCAAAACC
R-aro10-Int	ACAATTGGTAGCAGTGTTTTATAATTGCGCCCACAAGTTTGAGCGACCTCATGCTATACC
F-Delta-Int	TGTTGGAATAAAAATCCACTATC
R-Delta-Int	ATGGGGGTTCTCTGGAACAG
F-adh6v	ACAGCCACTCTCGTCACGGC
R-adh6v	CACCTTAAAGGTGCTTAGC
F-adh7v	GATACGTTTGGCTCTGTTGC
R-adh7v	CACTGTTGTCGAGAGATTC
F-sfa1v	AGACATGCGGTGTGTGGGTC
R-sfa1v	GTTAGGAACAGGCGAGGTC
F-gre2v	ACATTGTTGTACGCTATAG

R-gre2v	GCTTATCTGAACGTTTCTC
F-hfd1v	CTTAGAGGAAATGGAACAAC
R-hfd1v	GAAAGGTTACTTATACATC
BLH-BamHI-F	CGCGGATCCAAACAATGGGTTTAATGTTAATTGATTGG
BLH-XhoI-R	CCGCTCGAGTTAATTTTTAATTTTAATCCTTGAAGAATGTG
CrtE-BamHI-F	TTGGTCTCGGATCCAAACAATGGATTACGCGAACATCC
CrtE-XhoI-R	TTGGTCTCCTCGAGTCACAGAGGGATATCGGCTAG
CrtYB-BamHI-F	CGGGATCCAAACAATGACGGCTCTCGCATATTAC
CrtYB-XhoI-R	TTGGTCTCCTCGAGTTACTGCCCTTCCCATCCGC
CrtI-BamHI-F	CGGGATCCAAACAATGGGAAAAGAACAAGATCAG
CrtI-XhoI-R	TTGGTCTCCTCGAGTCAGAAAGCAAGAACACCAAC
ACL1_BamHI_F	GGGATCCAAAACAATGTCTGCCAACGAGAACATC
ACL1_SalI_R	AGACGCGTCGACTTATGATCGAGTCTTGGCC
ACL2_EcoRI_F	GGAATTCAAAACAATGTCAGCGAAATCCATTC
ACL2_XbaI_R	GCTCTAGATTAAACTCCGAGAGGAGTG

Table S2 Plasmids and strains used in this study.

Name	Description
Plasmid	
p415-GPD-Cas9	Plasmid harboring *Cas9* gene under the control of GPD promoter with LEU selection marker
pRS426SNR52	Plasmid harboring P_{SNR52}-T_{SUP4} cassette
pRS426-gRNA(adh6)	pRS426SNR52 derivative with P_{SNR52}-gRNA_adh6-T_{SUP4}
pRS426-gRNA(adh7)	pRS426SNR52 derivative with P_{SNR52}-gRNA_adh7-T_{SUP4}
pRS426-gRNA(sfa1)	pRS426SNR52 derivative with P_{SNR52}-gRNA_sfa1-T_{SUP4}
pRS426-gRNA(gre2)	pRS426SNR52 derivative with P_{SNR52}-gRNA_gre2-T_{SUP4}
pRS426-gRNA(hfd1)	pRS426SNR52 derivative with P_{SNR52}-gRNA_hfd1-T_{SUP4}
pRS426-gRNA(bts1)	pRS426SNR52 derivative with P_{SNR52}-gRNA_bts1-T_{SUP4}
pRS426-gRNA(yjl064w)	pRS426SNR52 derivative with P_{SNR52}-gRNA_yjl064w-T_{SUP4}
pRS426-gRNA(rox1)	pRS426SNR52 derivative with P_{SNR52}-gRNA_rox1-T_{SUP4}
pRS426-gRNA(ypl062w)	pRS426SNR52 derivative with P_{SNR52}-gRNA_ypl062w-T_{SUP4}
pRS426-gRNA(aro10)	pRS426SNR52 derivative with P_{SNR52}-gRNA_aro10-T_{SUP4}
pδBLE1.1[1]	Plasmid harboring P_{GAL1}-T_{CYC1} cassette
pδBLE-CrtE	pδBLE1.1::P_{GAL1}-*crtE*-T_{ADH1}
pδBLE-CrtYB	pδBLE1.1::P_{GAL1}-*crtYB*-T_{ADH1}
pδBLE-CrtI	pδBLE1.1::P_{GAL1}-*crtI*-T_{ADH1}
pRS426-gRNA(CR)[3]	pRS426SNR52 derivative with P_{SNR52}-gRNA_P_{GAL4}-T_{SUP4}, and P_{SNR52}-gRNA_P_{GAL80}-T_{SUP4}
pδBLE2.0-ERG13/ERG10[4]	pδBLE2.0::P_{GAL10}-*ERG13*-T_{ADH1}; P_{GAL1}-*ERG10*-T_{CYC1}
pδBLE2.0-ERG12/tHMG1[4]	pδBLE2.0::P_{GAL10}-*ERG12*-T_{ADH1}; P_{GAL1}-*tHMG1*-T_{CYC1}
pδBLE2.0-ERG19/ERG8[4]	pδBLE2.0::P_{GAL10}-*ERG19*-T_{ADH1}; P_{GAL1}-*ERG8*-T_{CYC1}
pδBLE2.0-IDI1/ERG20[4]	pδBLE2.0::P_{GAL10}-*IDI1*-T_{ADH1}; P_{GAL1}-*ERG20*-T_{CYC1}
pRS426-ACL	pESC-URA derivative with P_{GAL10}-*ACL1*-T_{ADH1}; P_{GAL1}-*ACL2*-T_{CYC1}
YEplac195-YB/I/E	Plasmid harboring *crtYB/I/E* gene from *X. dendrorhous*
pRS425TEF2-BLH	pRS425TEF2 derivative with P_{TEF2}-*BLH*-T_{CYC1}
pRS425GAL1-BLH	pRS425GAL1 derivative with P_{GAL1}-*BLH*-T_{CYC1}
Strain	
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
JS-M5	Strain BY4741derivative with Δ*adh6*, Δ*adh7*, Δ*sfa1*, Δ*gre2* and Δ*hfd1*
JS-WT-P	Strain BY4741 transformed with YEplac195-YB/I/E and pRS425TEF2-*BLH*
JS-M5-P	Strain JS-M5 transformed with YEplac195-YB/I/E and pRS425TEF2-*BLH*
JS-M5-CR	Strain JS-M5 derivative with copper-regulated expression system[3]
JS-AE	Strain JS-M5-CR derivative with Δ*bts1*:: P_{GAL1}-*crtE*-T_{CYC1}, Δ*ypl062w*:: P_{GAL1}-*crtYB*-T_{CYC1}, Δ*rox1*:: P_{GAL1}-*crtI*-T_{CYC1}
JS-AE1	One isolated variant derived from JS-AE with random integration of MVA modules under the control of GAL1/10 promoter
JS-AE2	One isolated variant derived from JS-AE with random integration of MVA modules under the control of GAL1/10 promoter
JS-AE3	One isolated variant derived from JS-AE with random integration of MVA

	modules under the control of GAL1/10 promoter
JS-BE	Strain JS-AE3 derivative with Δ*aro10*::P_{GAL10}-*ACL1*-T_{ADH1}; P_{GAL1}-*ACL2*-T_{CYC1}
JS-CE	Strain JS-AE3 derivative with Δ*yjl064w*::P_{GAL1}-*BLH*-T_{CYC1}
JS-CE1	Strain JS-BE derivative with Δ*yjl064w*::P_{GAL1}-*BLH*-T_{CYC1}

4 References

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4. J. F. Yuan and C. B. Ching, *Metab Eng*, 2016, **38**, 303-309.