Supplementary materials

Title: Improvement of cis,cis-muconic acid production in *Saccharomyces cerevisiae* through biosensor-aided genome engineering

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Figure S1. Fluorescence of yeast cells selected via biosensor-aided FACS. Individual ST8424 mutant cells sorted for the maximum (Max, a) or minimum (Min, b) fluorescence output were subjected to the fluorescence measurement on the overnight pre-culture of single colonies (a, b). The fluorescence of 109 out of 270 individual isolates from Mut_max (a) was higher than the control ST8424, of which 55 Mut_max isolates showed 20% higher fluorescence (a). Whereas, 81 out of 90 isolates from Mut_min was lower (b), of which 69 Mut_min isolates showed 60% lower fluorescence (b). The pre-culture of all the 55 Mut_max isolates and 10 randomly selected strains from the 69 Mut_min isolates were inoculated into fresh YPD medium for 24 h cultivation for the fluorescence measurement (c). 50/55 Mut_max isolates (c) showed higher fluorescence than the control strain ST8424 and 10/10 Mut_min isolates (c) showed lower fluorescence than the control strain ST8424. 42 Mut_max strains out of the 50 isolates showing 25% higher fluorescence and 4 Mut_min strains out of the 10 isolates showing 70% lower fluorescence were selected for the subsequent cis,cis-muconic acid (CCM) production test. Data shown are mean values ± SDs of triplicates for control strains (ST8424, ST8425) or single replicates for individual mutant strains.
Figure S2. Concentrations of CCM and protocatechuic acid (PCA) in the fermentation broth of control strain ST8424 and its mutants after 72 hours in YPD medium.
Forty-two mutant strains with the highest fluorescence output from the 24 h culture were evaluated for CCM production with 72h culture on YPD medium. Data shown are mean values ± SDs of triplicates for control strains (ST8424, ST8425) or single replicate for individual mutant strains. Arrows indicate the mutant strains with improved CCM production which we have further validated by cultivation on two other types of media.
Figure S3. Concentrations of CCM and PCA in the fermentation broth of control strain ST8424 and its mutants after 72 hours in mineral medium (MM) and mimicked fed-batch/feed-in-time medium (FIT).

Eight mutant strains, which showed significantly improved CCM production in YPD medium, were evaluated on the CCM production in MM and FIT media. Data shown are mean values ± SDs of triplicates.
Figure S4. Characterization of duplication of engineered genes in Mut131
Duplication of the engineered genes was analyzed by comparing the read coverage of heterologous genes. The artificial reference sequence used in this analysis contains all heterologous genes in STB424 as well as native *S. cerevisiae* TKL1 which serves as an indicator for two-copy genes.
Figure S5. Effect of uracil supplementation on CCM production by *S. cerevisiae*
The two URA3-deficient *S. cerevisiae* strains, ST8424 and ST8920, were cultivated on mineral medium with uracil supplementation at various levels for 72 h. The 72 h cell culture was then subjected to the quantification of Optical density at 600 nm (OD$_{600}$), CCM production and specific CCM production.
Data shown are mean values ± SDs of triplicates.
Figure S6. Optical density of engineered strains cultivated on FIT medium for 72 hours. Data shown are mean values ± SDs of triplicates.
Figure S7. CCM toxicity to *S. cerevisiae* under different pH.

Cell growth of CEN.PK113-7D strain was monitored over 72 h on mineral medium supplemented with CCM at various levels, under the conditions that initial pH was unadjusted (a) or adjusted (to 6.0, b). In tests with ≥ 5 g/L CCM supplementation and initial pH unadjusted, CCM is largely in the insoluble form, whereas, it is all soluble in the rest tests. pH of mixed culture of biological triplicates (c) was measured after 72 h cultivation.

WO: without; W: with. Media without cells were used as the control for calibration.

Data shown in panel a and b are mean values ± SDs of triplicates.

| MM with muconic acid supplement (g/L) | pH unadjusted initially | pH adjusted initially to 6.0 | WO cells | W cells | WO cells | W cells |
|-------------------------------|------------------------|----------------------------|----------|--------|----------|--------|
| 0.1                           | 5.91                   | 3.69                      | 5.94     | 2.98   |          |        |
| 0.5                           | 5.82                   | 3.06                      | 5.77     | 2.68   |          |        |
| 1                             | 5.58                   | 2.88                      | 5.83     | 3.38   |          |        |
| 5                             | 5.31                   | 3.43                      | 5.98     | 4.95   |          |        |
| 10                            | NA*                    | NA                        | 5.88     | 5.01   |          |        |
| 50                            | NA                     | NA                        | 5.78     | 5.42   |          |        |

*: NA: not applied as no cell growth in these cases.
Figure S8. Intracellular CCM concentrations from 72h cultivation of CEN.PK 113-7D fed with CCM, and strains engineered to produce CCM.

Yeast strains are cultured on mineral medium (initial pH 6.0) with (CEN.PK 113-7D) or without (ST8424 and ST8920) CCM supplementation. Data shown are mean values ± SDs of triplicates.
Figure S9. Controlled fed-batch fermentation for CCM production. Fermentation BD1 (a) and BD2 (b) of ST8943 strain was performed by feeding different feeding solution into 1.3 L starting fermentation broth. BD1 was fed with 289 mL of 800 g/L glucose (a) and BD2 was fed with 285 mL of 800 g/L glucose and 4 g/L KH$_2$PO$_4$ (b). Data shown are from single replicate.
Figure S10. Salt concentration and fermentation parameters during the controlled fed-batch fermentations BD1 and BD2.
Figure S11. Salt concentration and fermentation parameters during the controlled fed-batch fermentations BD3, BD4 and BD5.
Figure S12. Salt concentration and fermentation parameters during the controlled fed-batch fermentation BD6.
Table S1. Mutation in the intergenic region in Mut131

| Mutations*       | Intergenic location | Detail |
|------------------|---------------------|--------|
| SNP_chr01_176020 | YAR019W-A-ARS10     | G->A   |
| SNP_chr02_728740 | RIB5-POP4           | C->T   |
| SNP_chr02_728769 | RIB5-POP4           | A->T   |
| SNP_chr04_1337854| GPI19-THI74         | C->T   |
| SNP_chr04_1521095| YDR514C-PAU10       | C->T   |
| SNP_chr04_790149 | TAF10-CDC37         | G->A   |
| SNP_chr07_848453 | ERG1-YGR176W        | T->C   |
| SNP_chr07_875775 | YGRWdelta31         | C->A   |
| SNP_chr14_552680 | COG6-YNL040W        | C->T   |

*: genomic location is based on the S288C genome.
Table S2. The concentrations of PCA and CCM in the fermentation broth of engineered *S. cerevisiae* strains after 72-hour cultivation in FIT medium

| Strains | PCA+CCM (mg/L) | PCA+CCM (mg/OD$_{600}$/L) |
|---------|----------------|---------------------------|
| ST8424  | 501.84±8.67    | 105.71±1.88               |
| ST8918  | 1027.53±34.07  | 184.19±6.10               |
| ST8919  | 1020.46±6.59   | 193.90±3.25               |
| ST8920  | 1047.66±11.08  | 257.92±3.24               |
| ST8942  | 1759.07±5.01   | 270.66±5.77               |
| ST8943  | 1934.86±51.62  | 303.10±14.29              |
Table S3. Strains used in this study

| Strains               | Genotype                                                                 | Reference/source |
|-----------------------|--------------------------------------------------------------------------|------------------|
| CEN.PK113-7D          | MAT a URA3 HIS3 LEU2 TRP1                                                 | 1                |
| CEN.PK102-5B          | MATa ura3-52 his3Δ1 leu2-3/112 MAL2-8c SUC2 CEN.PK102-5B X-4-HIS5-ScTKL1-KpAroY.D-XI-1-KILEU2-PaAroZ-CaCatA | 1                |
| ST2377                |                                                                          | 2                |
| ST8420                | ST2377-XII-2-amdSYM-Cas9                                                 | This study       |
| ST8421                | ST8420-X-2-KpAroY.B-KpAroY.Ciso                                          | This study       |
| ST8422                | ST8420-X-2-KpAroY.B-KpAroY.Ciso-XI-5-KpAroY.B-KpAroY.Ciso                | This study       |
| ST8423                | ST8420-X-2-KpAroY.B-KpAroY.Ciso-XI-5-KpAroY.B-KpAroY.Ciso                | This study       |
| ST8424                | ST8421-X-3-REV1p-BenM variant MP02_D04-XI-2-CYC1p-yEGFP                  | This study       |
| ST8425                | ST8422-X-3-REV1p-BenM variant MP02_D04-XI-2-CYC1p-yEGFP                  | This study       |
| ST8426                | ST8423-X-3-REV1p-BenM variant MP02_D04-XI-2-CYC1p-yEGFP                  | This study       |
| ST8918/Mut131         | ST8424 UV_mutations                                                      | This study       |
| ST9124                | ST8424 MNE1_Lys450Glu                                                    | This study       |
| ST9286                | ST8424 MNE1_Lys450Glu CDC15_Pro429Phe                                   | This study       |
| ST9288                | ST8424 MNE1_Lys450Glu DIT1_Glu526Gly                                      | This study       |
| ST9657/RC1            | ST8424 XII-5_60bp-PaAroZ-CaCatA                                          | This study       |
| ST9658/RC2            | ST9286 XII-5_60bp-PaAroZ-CaCatA                                          | This study       |
| ST9659/RC3            | ST9288 XII-5_60bp-PaAroZ-CaCatA                                          | This study       |
| ST8919                | ST8918-XI-5-KpAroY.B-KpAroY.Ciso                                         | This study       |
| ST8920                | ST8918-XI-5-KpAroY.B-KpAroY.Ciso-XII-4-KpAroY.B-KpAroY.Ciso              | This study       |
| ST8942                | ST8920-XI-3-KIURA3                                                       | This study       |
| ST8943                | ST8920-XI-3-ScARO4-ScARO1deltaAroE-KIURA3                                | This study       |
| Plasmid       | Feature                                                                 | Parental plasmid | DNA insert                                                                 | Note                                                                 | Construction approaches/reference |
|--------------|-------------------------------------------------------------------------|------------------|---------------------------------------------------------------------------|----------------------------------------------------------------------|-----------------------------------|
| pCFB2314     | XII-2-TEF1p-CAS9-amdSYM 2µ ori, AMPPr, gRNA X-2                         | NA               | NA                                                                        | Genome integration of Cas9                                           | 3                                 |
| pCFB3020     | NatMXsyn gRNA X-2                                                       | NA               | NA                                                                        | gRNA plasmid for X-2 integration                                     | 4                                 |
| pCFB3046     | NatMXsyn gRNA XI-5                                                      | NA               | NA                                                                        | gRNA plasmid for XI-5 integration                                     | 4                                 |
| pCFB3053     | NatMXsyn gRNA X-2 XI-5 XII-4                                           | NA               | NA                                                                        | gRNA plasmid for simultaneous integration into X-2, XI-5 and XII-4 sites | 4                                 |
| pCFB3051     | gRNA X-3, XI-2, XII-2 XII-4-CYC1p (BenM Binding Sits)-yEGFP-loxP-Hph    | NA               | NA                                                                        | Template for integrative cassette (XI-2-CYC1p-yEGFP ) construction    | 2                                 |
| pCFB2696     | XI-3-KlURA3-KpAroY.B-KpAroY.Ciso                                        | NA               | NA                                                                        | Template for integrative cassettes (X-2-AroY.B-Ciso, XI-5-AroY.B-Ciso, XII-4-AroY.B-Ciso) construction | 2                                 |
| pCFB7731     | MP2_D04 isolated from yeast (BenM variant MP02_D04)                     | NA               | NA                                                                        | Template for integrative cassette (X-3-REV1p-BenM variant MP02_D04 ) construction | 5                                 |
| pCFB390      | ori, AMP, XI-3-LoxP-KlURA3                                              | NA               | NA                                                                        | Template for backbone (ori-AMPPr-XI-3-KlURA3) for pCFB8808            | 6                                 |
| pCFB2899     | ori, AMP, X-2                                                           | NA               | NA                                                                        | Backbone for pCFB8084                                                | 4                                 |
| pCFB8084     | ori, AMP, X-2-TEF1p-ScARO4-PGK1-ScARO1deltaAroE                         | pCFB2899 ScARO4, ScARO1deltaAroE, TEF1p-PGK1p | Template for insert (TEF1p-ScARO4-PGK1-ScARO1deltaAroE) for pCFB8808 | USER Cloning                                                                  |
pCFB8808 ori, AMP, XI-3- TEF1p-ScARO4- PGK1- ScARO1deltaAroE-KIURA3

pCFB390 TEF1p- ScARO4- PGK1p- ScARO1deltaAroE- KIURA3

Genome integration of ScARO4- ScARO1deltaAroE- KIURA3

p0029(pSP- GM1) 2µ ori, URA3, Amp NA NA Control plasmid for effect evaluation of point mutation pME10 derived plasmid, used as template for backbone preparation for the plasmid via PCR, for the mutation introduction

pQC003/pCFB8904 2µ ori, KIURA3, Amp, GAL80 gRNA NA NA Requested from Chalmers University of Technology

pCFB8898 2µ ori, KIURA3, Amp, PWP2 gRNA-165 bp disrupt pCFB8904 PWP2-dis Point mutation introduction into PWP2

pCFB8886 2µ ori, KIURA3, Amp, ATG1 gRNA-165 bp disrupt pCFB8904 ATG1-dis Point mutation introduction into ATG1

pCFB8896 2µ ori, KIURA3, Amp, PET54 gRNA-165 bp disrupt pCFB8904 PET54-dis Point mutation introduction into PET54

pCFB8900 2µ ori, KIURA3, Amp, SAP1 gRNA-165 bp disrupt pCFB8904 SAP1-dis Point mutation introduction into SAP1

pCFB8897 2µ ori, KIURA3, Amp, PUT3 gRNA-165 bp disrupt pCFB8904 PUT3-dis Point mutation introduction into PUT3

pCFB8893 2µ ori, KIURA3, Amp, NUD1 gRNA-165 bp disrupt pCFB8904 NUD1-dis Point mutation introduction into NUD1

pCFB8891 2µ ori, KIURA3, Amp, KNS1 gRNA-165 bp disrupt pCFB8904 KNS1-dis Point mutation introduction into KNS1

pCFB8887 2µ ori, KIURA3, Amp, CDC15 gRNA-165 bp disrupt pCFB8904 CDC15-dis Point mutation introduction into CDC15

pCFB8892 2µ ori, KIURA3, Amp, MNE1 pCFB8904 MNE1-dis Point mutation introduction into MNE1

pCFB8898 2µ ori, KIURA3, Amp, PWP2 gRNA-165 bp disrupt Gibson assembly

pCFB8886 2µ ori, KIURA3, Amp, ATG1 gRNA-165 bp disrupt Gibson assembly

pCFB8896 2µ ori, KIURA3, Amp, PET54 gRNA-165 bp disrupt Gibson assembly

pCFB8900 2µ ori, KIURA3, Amp, SAP1 gRNA-165 bp disrupt Gibson assembly

pCFB8897 2µ ori, KIURA3, Amp, PUT3 gRNA-165 bp disrupt Gibson assembly

pCFB8893 2µ ori, KIURA3, Amp, NUD1 gRNA-165 bp disrupt Gibson assembly

pCFB8891 2µ ori, KIURA3, Amp, KNS1 gRNA-165 bp disrupt Gibson assembly

pCFB8887 2µ ori, KIURA3, Amp, CDC15 gRNA-165 bp disrupt Gibson assembly

pCFB8892 2µ ori, KIURA3, Amp, MNE1 Gibson assembly

Requested from Chalmers University of Technology
| Vector | Constructs | gRNA-165 bp disrupt | Disrupted Gene | Point mutation introduction into | Assembly Method |
|--------|------------|---------------------|---------------|---------------------------------|----------------|
| pCFB8894 | pCFB88904 | 2µ ori, KIURA3, Amp, NUP53 | NUP53-dis | NUP53 | Gibson assembly |
| pCFB8890 | pCFB88904 | 2µ ori, KIURA3, Amp, EST2 | EST2-dis | EST2 | Gibson assembly |
| pCFB8899 | pCFB88904 | 2µ ori, KIURA3, Amp, RET2 | RET2-dis | RET2 | Gibson assembly |
| pCFB8895 | pCFB88904 | Amp, OCA5 | OCA5-dis | OCA5 | Gibson assembly |
| pCFB8889 | pCFB88904 | 2µ ori, KIURA3, Amp, DIT1 | DIT1-dis | DIT1 | Gibson assembly |
| pCFB8888 | pCFB88904 | 2µ ori, KIURA3, Amp, CTS2 | CTS2-dis | CTS2 | Gibson assembly |
| pCFB1239 | NA | XI-1-LoxP-KLEU2-PaAroZ-PTDH3-PTEF1->CaCatA | NA | Template for integrative cassette | (Skjoedt et al., 2016) |
Table S5. Synthetic DNA fragment sequences

| Name     | Sequence                                                                 |
|----------|--------------------------------------------------------------------------|
| PWP2-dis | CTGTACAGCTAACTGAAGGtttttagctagaataatagcaagtttaaatagctagttcctgcttcactatggaa  |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| ATG1-dis | ATCAATGGCAGATAATCCCGgttttagagctagaataatagcctagttcctgcttcactatggaa         |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| PET54-dis| CAGTCATCTCAGTCAACTGGCCACgttttagagctagaataatagcctagttcctgcttcactatggaa     |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| SAP1-dis | CAGTACAGCTAACTGAAGGtttttagctagaataatagcaagtttaaatagctagttcctgcttcactatggaa  |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| PUT3-dis | CAATGTTCCGATTTTACTTTACTTTcttttagagctagaataatagcctagttcctgcttcactatggaa   |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| NUD1-dis | CAGGACGACGCAATGCAACTGGCCACgttttagagctagaataatagcctagttcctgcttcactatggaa     |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| KNS1-dis | GCGGCATGGATTTACTTTACTTTACTTTcttttagagctagaataatagcctagttcctgcttcactatggaa  |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| CDC15-dis| CAGACCAAAATATGCTGATTTACTTTACTTTACTTTcttttagagctagaataatagcctagttcctgcttcactatggaa  |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |
| MNE1-dis | ATATGTTGCTGCTACGATTTTTTTTTTTTTacttttagagctagaataatagcctagttcctgcttcactatggaa  |
|          | aagttgccgaaggactggctgttcctttttatatctgtCTAGTGACAGCATCCGAAGATGGGAAAATCA     |
|          | AAGTTTGGGAAGTGAAGATGTGTTGTTCTCACTCATCGTTAGATGTGAGGTGAGCTGCTGAGCCTGCTGAGCCTG |
|          | ATGGGAAAATCA                                                             |

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attccacGGGCAGCTCTCAAGATTTTCTACCAAACCTACTCTACTGCGATTTGATAACCCAGGTGAA
TGAATTCGGCCTATC

NUP3-dis

GATCCGCCTCTACTGTGCTGTTTtagagctagaatagaagtttaaataaagctagtccgttatcaactttga
agttggcaccagctgcgtgtctttttttttttttttgtcAGCAAGGACGCAAAAGAGCCAAACGGGACTG
TTAAAGGTTTAAACCGGTTTCCAGCTGACCAAAACCGCCTTCTTATTGGAaatcaccacaagttaca
/gttaaacGAACCTGAATGATAATCCAGCGTGGTTCAATAATCCAAGGAAAAGAGCCATCTCAAAT
CACAATAAAGGAGATTA

EST2-dis

TTTGATTTGCGCAGCAGTTGgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaactttga
agttggcaccagctgcgtgtctttttttttttttttgtcATTTGAGTAGCTATTATAGCTCCATCAAAAGAAATA
CACAAGTTGTTGTTTGCTGCTTCTTATCTACGCGTCAGGGGAAAAtctttgcttagtcgacagggtaa
GGAGGACATTTCGTTATCAAGAAAATCTCTTAATAAAGTAAGAGCGCTACATTGGTCTACCTTT
TTTGTTCTTTTACTTAAA

RET2-dis

CGAAGTCGGTAATGATGCTAgttttagagctagaatagaagtttaaataaagctagtccgttatcaactttga
agttggcaccagctgcgtgtctttttttttttttttgtcACGAACTTACCTGCGACCAAAATGGGCTCAA
GCTGACCATCCTCATCAagcgctacagcagcacagata

OCA5-dis

ACTCGTTTCGATATTGGAGAgttttagagctagaatagaagtttaaataaagctagtccgttatcaactttga
agttggcaccagctgcgtgtctttttttttttttttgtcACGAACTTACCTGCGACCAAAATGGGCTCAA
GCTGACCATCCTCATCAagcgctacagcagcacagata

DIT1-dis

TTGAATAAAATGGGCATGGGgttttagagctagaatagaagtttaaataaagctagtccgttatcaactttga
agttggcaccagctgcgtgtctttttttttttttttgtcACGAACTTACCTGCGACCAAAATGGGCTCAA
GCTGACCATCCTCATCAagcgctacagcagcacagata

Note: The fragments synthesized by Twist Bioscience contain two adaptor at 5' (GAAGTGCCATTCCGCCTGACCT) and 3' (CCTGACATTCCACCTAGGCCT) ends respectively, which were used for the plasmid cloning through Gibson assembly
| Primers | Sequence | Note | Purpose |
|---------|----------|------|---------|
| 22744   | cgtctatgaggagacctgttagttgg | Flanking region for homologous recombination | Construction of X-2-AroY.B-Ciso via overlapping PCR |
| 22745   | caagttgcctgcaagtttctctg |  |  |
| 22746   | cagagaaactgcagggcaacttggttagttgcatctctag | Fragment of AroY.B-Ciso expression cassette |  |
| 22747   | cccctctgagggcaggtcattccatcaagctacagtata |  |  |
| 22748   | cctgcataatcggcctcacag | Fragment of AroY.B-Ciso expression cassette |  |
| 22749   | cccgtgtgaggccgattatgcaggttctcaagcaaggtttacctgata |  |  |
| 22750   | ggcggagaagttggtagatagcattcc | Flanking region for homologous recombination |  |
| 22751   | tgtgtcagggagtttagctgcacca |  |  |
| 22752   | tgtgcataaatcggcctcacag | Fragment of AroY.B-Ciso expression cassette |  |
| 22753   | gggagtactatgaagccagccaatattctcaagcaaggtttacctgata |  |  |
| 22754   | tatgggtctctcatagtcacc | Flanking region for homologous recombination |  |
| 22755   | gatcatagatccggcacttagagaga |  |  |
| 22756   | gatccggctgtttccattagcc | Flanking region for homologous recombination |  |
| 22757   | tgcctagatagtgtgtgtagggaaatttccatcacatacatatggcagcagctacctgcatctctactgaggttctcaagcaaggtttacctgata | Fragment of AroY.B-Ciso expression cassette |  |
| 22758   | ttctttatttgactctaatggggaatttctcaagcaaggtttacctgata |  |  |
| 22759   | ATTCGCCATTTAGAGTCAAATAAAG | Flanking region for homologous recombination |  |
| 22760   | TTTCCTGCTGTACCTGGATGGTGC |  |  |
| 22761   | cgcagatccttgggttccgattacc | Flanking region for homologous recombination |  |
| 22762   | agtctctgtatgcgctccgtcgc |  |  |
| 22763   | ggcagagcgcagcagactttttagtgcagcaacattttcatattcttcat | Fragment of REV1p-BenM variant MP02_D04 expression cassette |  |
| 22764   | caatatcgtttcattgaaagttggttcctttcaagcaaggtttccagtataaa |  |  |
| 22765   | cccttttcatgaagccagctatttg | Flanking region for homologous recombination |  |
| 22766   | gaggtggttagattgatcaccggaa |  |  |
| 22767   | ggggagctttcgtgaggagcagagtag | Flanking region for homologous |  |
| 22768   | tggagactttcgtgaggtggttgtgcttcggattacc |  |  |
| 22769   | ATTCGCCATTTAGAGTCAAATAAAG |  |  |
| 22770   | TTTCCTGCTGTACCTGGATGGTGC |  |  |
| 22771   | cgcagatccttgggttccgattacc | Flanking region for homologous recombination |  |
| 22772   | agtctctgtatgcgctccgtcgc |  |  |
| 22773   | ggcagagcgcagcagactttttagtgcagcaacattttcatattcttcat | Fragment of REV1p-BenM variant MP02_D04 expression cassette |  |
| 22774   | caatatcgtttcattgaaagttggttcctttcaagcaaggtttccagtataaa |  |  |
| 22775   | cccttttcatgaagccagctatttg | Flanking region for homologous recombination |  |
| 22776   | gaggtggttagattgatcaccggaa |  |  |
| 22777   | ggggagctttcgtgaggagcagagtag | Flanking region for homologous |  |
| 22778   | tggagactttcgtgaggtggttgtgcttcggattacc |  |  |

Table S6. Primers used in this study
| Sequence | Description |
|----------|-------------|
| gagcgtttgccatgaacctccaacaggcaacttttag tctgacaca aatatctgaaacgctagtctgctgtttgcagctcaca aacctttccaa cacagactagcgtttcagatatt | Fragment of CYC1p-yEGFP expression cassette |
| gtgggaagattccgctctc | Flanking region for homologous recombination |

| Sequence | Description |
|----------|-------------|
| AGTGCAGGUAAAAAACATGGAATCTCC AATGTTCG CGTGCGAUTCATTCTTTGTAACCTTCTTCTTTG | ScARO4 amplified from pCFB775 |
| ATCTGTCAUUAAAAACATGGAATCTTCTTTGAAAG | ScARO1deltaAroE amplified from genome |
| CACGCGAUTCATTCTTTGTAACCTTCTTCTTCTTTG | pCFB8804 construction |
| acgtgcacutttataaaacttag atgacagautttatatattgttg | TEF1p-PGK1p amplified from p0029 |

| Sequence | Description |
|----------|-------------|
| ATCGCGTGCAUTCATTGCTACTGTCAATTGG | Aro4-TEF1p-PGK1p-Aro1deltaAroE |

| Sequence | Description |
|----------|-------------|
| ATCGCGTGCAUTCATTGCTACTGTCAATTGG | Backbone with elements of Amp-XI-3-KlURA3 |
| acgtgcacutttataaaacttag atgacagautttatatattgttg | Aro4-TEF1p-PGK1p-Aro1deltaAroE |

| Sequence | Description |
|----------|-------------|
| AGGTCAAGGGGAATGGCAGCTTCgatcattttactttcactgcggagaag | Plasmid construction for mutation introduction |
| AGGTCAAGGGGAATGGCAGCTTCgatcattttactttcactgcggagaag | Backbone of plasmid for mutation introduction |

| Sequence | Description |
|----------|-------------|
| GACTACGTTATGGGTCGTTCTTCA | PCR product from transformants for PWP2 mutation |
| GACTACGTTATGGGTCGTTCTTCA | Transformant validation for mutation introduction via sequencing of the |
| CCATGATGACAGAAGCTAAGACAC | Sequencing primer of PWP2 PCR fragment |
| TTCCAGCAAACTTGGGCAATAG | PCR product from transformants for ATG1 mutation |

| Sequence | Description |
|----------|-------------|
| TACCCTCCACGGAGTGAAGACC | PCR product from transformants for ATG1 mutation |

| Sequence | Description |
|----------|-------------|
| 7171 | pCFB8808 construction |

| Sequence | Description |
|----------|-------------|
| 23260 | Plasmid construction for mutation introduction |
| 23261 | Backbone of plasmid for mutation introduction |

| Sequence | Description |
|----------|-------------|
| 24309 GACTACGTTATGGGTCGTTCTTCA | Transformant validation for mutation introduction via sequencing of the |
| 24310 CCATGATGACAGAAGCTAAG | Sequencing primer of PWP2 PCR fragment |
| 24311 TTCCAGCAAACTTGGGCAATAG | PCR product from transformants for ATG1 mutation |
| Line | Sequence | Description |
|------|----------|-------------|
| 24314 | TGTGGCACCCCAACGTTTAG | Sequencing primer of ATG1 PCR fragment |
| 24315 | CCTGTTTCTATGGAGACACACCC | PCR product from transformants for PET54 mutation |
| 24316 | CGTGCAGCCTAATTAGTGG | Sequencing primer of PET54 PCR fragment |
| 24317 | ATACTAACACGCACTCAATAG | PCR product from transformants for PET54 mutation |
| 24318 | CCAAGAAGTACTACATACGCGTCG | Sequencing primer of PET54 PCR fragment |
| 24319 | CTACGTCGACTTCGGAGAAATTATCA | PCR product from transformants for SAP1 mutation |
| 24320 | CTTTACCTCGTTGTTTACC | Sequencing primer of SAP1 PCR fragment |
| 24321 | GTAGTGGCAGGAAGAGGTATACATACA | PCR product from transformants for PUT3 mutation |
| 24322 | GACGAAAGTATGTCTGTTGAACC | Sequencing primer of PUT3 PCR fragment |
| 24323 | ATGAAATCGAGCATCATAGGAGAC | PCR product from transformants for PUT3 mutation |
| 24324 | ATGGCAGACCTACAAAAACAGGAG | Sequencing primer of PUT3 PCR fragment |
| 24325 | GTCAACTTGACCATCCATCTCC | PCR product from transformants for NUP53 mutation |
| 24326 | CGAATCACAGGGACAACATGAA | Sequencing primer of NUP53 PCR fragment |
| 24327 | AGGTATACCTCGAACGTTGTACA | PCR product from transformants for NUD1 mutation |
| 24328 | TTACAGATTGCCAGTGGG | Sequencing primer of NUD1 PCR fragment |
| 24329 | GAGACTTCACACACTTACCAGTG | PCR product from transformants for NUD1 mutation |
| 24330 | GGAACAGTGCTAGACCTGAATCAAGC | Sequencing primer of NUD1 PCR fragment |
| 24331 | GCAATGGCCTGAATATGAGAGCC | PCR product from transformants for KNS1 mutation |
| 24332 | GCAGCCAATTCAATTTTCGTC | Sequencing primer of KNS1 PCR fragment |
| 24333 | TCAGCGACATTGCTCTAGGTTG | PCR product from transformants for CDC15 mutation |
| 24334 | AAGAAGGAATAGCGGACTTGTGT | Sequencing primer of CDC15 PCR fragment |
| 24335 | ATCAAGAAGTATTTATGCC | PCR product from transformants for CDC15 mutation |
| 24336 | GGAGCTTGCTGAATGGAACCTTTCA | PCR product from transformants for MNE1 mutation |
| 24337 | CTCTCTTGCATAGGATACATCCGC | Sequencing primer of MNE1 PCR fragment |
| 24338 | AAGTGATTTGCCTCTGAACCTAGAA | |
| 24339 | CGAGTTCCATTCAAGACAACGGCTTCGAC | PCR product from transformants for EST2 mutation |
| 24340 | CAGAAATGAACACACCCAGAAAATC | Sequencing primer of EST2 PCR fragment |
| 24341 | ATTCTGCAAATGTTACGTTACGT | |
| 24342 | CAGGGCAAGTAAAGTGGATTGCGG | PCR product from transformants for RET2 mutation |
| 24343 | CTAGCAATTTTCTTAGCACGCGC | Sequencing primer of RET2 PCR fragment |
| 24344 | CATTTTGGATCTTGACGTTTCC | |
| 24345 | GACTAGCTTTGCTATTCTCACATTG | PCR product from transformants for OCA5 mutation |
| 24346 | TCAAGAGAGCTTTCTCTAATCTT | Sequencing primer of OCA5 PCR fragment |
| 24347 | TGATAAGTCAGAATTTGAAACTACG | |
| 24348 | AGAATTGAATGCATTTGCCAGC | PCR product from transformants for DIT1 mutation |
| 24349 | CTTAGACAATGGCCATTGCA | Sequencing primer of DIT1 PCR fragment |
| 24350 | GCGTATTCGAACCTTAGTGAAT | |
| 24351 | GCAATCTATTCAGTGAGACGGAATT | PCR product from transformants for CST2 mutation |
| 24352 | GTGGATCATATGGGGCAACGTAT | Sequencing primer of CST2 PCR fragment |
| 24353 | GGTACAATAGAACACATAACGATCCAA | |

**Construction of XII-5_60bp-PaAroZ-CaCatA via PCR**

| 23820 | CCGGTACCGGAGGACCGGCTATAACCCGGTTGAATTTATTGTCAACAGTGCAATCCACGCGAGGACCTCATGCTATACCTGAGACTAAAACAATAAGGCTAGTTCGAATGATGAACTTGCTTGCTGTCAAACTTCTGAGTTGCCAATCTGATTTGCTCAAATCTCTGAGTTGCC | Fragment of PaAroZ-CaCatA expression cassette with two 60 bp homology arms for genome integration into XII-5 site |
Primer sets for genomic validation for gene integration can be found in (4)
Supplementary materials and methods

Medium

In the test of the uracil effect on CCM production, mineral media (pH 6.0) supplemented with 20, 50 or 100 mg/L uracil were used for the strain cultivation.

pH measurement

The pH of the 72 h culture cultivated using growth profiler was measured using the pH meter (Metrohm 827 pH lab). The replicate samples for each CCM treatment group were mixed together and the supernatant was used for the measurement.

Quantification of the intracellular CCM and PCA

Intracellular CCM and PCA was extracted from 500 μL of 72 h culture. The cell culture was first washed with 1 mL water and resuspended in 400 μL water for heating at 95 °C for 1 h and vortexing for 30 min. The supernatant was then obtained by centrifugation at 13,000 rpm for 3 min. The supernatant was analyzed using HPLC and CCM and PCA were quantified as described in ‘Metabolite quantification’ section in the main text.
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