## Supporting Information

### Table S1. *P. pastoris* strains used in this study

| Strain | Description | Reference or source |
|---|---|---|
| GS115 | *P. pastoris* strain, Mut$^+$ | Invitrogen, USA |
| SHY169 | *P. pastoris* strain with integrated cellulase gene (a codon-optimized endo-β-D-1,4-glucanase gene stce1 from *Staphylotrichum coccosporum*) in the genome, derived from GS115, Mut$^+$ | Wuhan Sunhy Biology Co., Ltd |
| R1-3 | mutant strain from the first round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain SHY169 | This study |
| R2-9 | mutant strain from the second round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R1-3 | This study |
| R3-4 | mutant strain from the third round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R2-9 | This study |
| R3-19 | mutant strain from the third round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R2-9 | This study |
| R3-24 | mutant strain from the third round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R2-9 | This study |
| R4-5 | mutant strain from the fourth round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R3-4 | This study |
| R4-6 | mutant strain from the fourth round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R3-4 | This study |
| R4-12 | mutant strain from the fourth round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R3-4 | This study |
| R5-2 | mutant strain from the fifth round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R4-6 | This study |
| R5-14 | mutant strain from the fifth round of ARTP mutagenesis of *P. pastoris* SHY169 and directly derived from the strain R4-6 | This study |
| SHY169 (rsc1Δ) | SHY169 rsc1 deletion strain, rsc1Δ::hphB | This study |
| SHY169 (rvs167Δ) | SHY169 rvs167 deletion strain, rvs167Δ::hphB | This study |
| SHY169 (pmt6Δ) | SHY169 pmt6 deletion strain, pmt6Δ::hphB | This study |
| R1-3 (rsc1Δ) | R1-3 rsc1 deletion strain, rsc1Δ::hphB | This study |
| R3-24 (rvs167Δ) | R3-24 rvs167 deletion strain, rvs167Δ::hphB | This study |
| R5-2 (pmt6Δ) | R5-2 pmt6 deletion strain, pmt6Δ::hphB | This study |

## Table S2. Primer used in this study

| Name | Sequence (5' → 3') Application | Application |
|---|---|---|
| HyhB-F | CACATCCGAACATAAACAACCATGAAAAAGCCTGAACTCACCG | Amplify hphB gene |
| HyhB-R | CTTTTTATTGTCAGTACTGACTATTCCTTTGCCCTCGGACG | Amplify hphB gene, reverse primer for verifying gene deletion using hphB expression cassette |
| Ptef-F | GACATGGAGGCCCAGAATACC | Amplify *S. cerevisiae* TEF1 promoter |
| Ptef-R | GGTTGTTTATGTTCGGATGTGATG | Amplify *S. cerevisiae* TEF1 promoter |
| Ttef-F | TCAGTACTGACAATAAAAAGATTCTTG | Amplify *S. cerevisiae* TEF1 terminator |
| Ttef-R | CAGTATAGCGACCAGCATTCACATAC | Amplify *S. cerevisiae* TEF1 terminator |
| RSC-HR1-F | CATACAGATTGACTGGTGCCGATAAC | Amplify 1 kb homologous sequence upstream *RSC1* |
| RSC-HR1-R | GTATTCTGGGCCTCCATGTCCAGAGATCAGCGTATTTCGTGTTTG | Amplify 1 kb homologous sequence upstream *RSC1* |
| RSC-HR2-F | GAATGCTGGTCGCTATACTGATAGGTCGATTCTTGTATATTGTAACAAT | Amplify 1 kb homologous sequence downstream *RSC1* |
| RSC-HR2-R | AAGTTACGATACGAAGAATTGAGAAGAAG | Amplify 1 kb homologous sequence downstream *RSC1* |
| RSC1-F | GTCGGAGGCTTTCAAGGAAG | Verify *RSC1* deletion |
| RSC1-R | GATTCTTACCTTCTGTAGGGACATCG | Verify *RSC1* deletion, negative control |
| RVS-HR1-F | CACGCTTGTATTTATGTCTGTACAAAGG | Amplify 1 kb homologous sequence upstream *RVS167* |
| RVS-HR1-R | GTATTCTGGGCCTCCATGTCATCGGATAGTTCGCAATTGAGG | Amplify 1 kb homologous sequence upstream *RVS167* |
| RVS-HR2-F | GAATGCTGGTCGCTATACTGTGCTAAATGTCAAATATCAAGAATCTATACG | Amplify 1 kb homologous sequence downstream *RVS167* |
| RVS-HR2-R | ACAATTAATGATCTCATGTCTGACGTG | Amplify 1 kb homologous sequence downstream *RVS167* |
| RVS167-F | CAGAGGAATAAGTGGCTCTGATAGTG | Verify *RVS167* deletion |
| RVS167-R | CTGAATCCAATTTGATGGTTCAAC | Verify *RVS167* deletion, negative control |
| PMT-HR1-F | CACTTGATTATCTAGGAAAGGCATTATC | Amplify 1 kb homologous sequence upstream *PMT6* |
| PMT-HR1-R | GTATTCTGGGCCTCCATGTCCTCGGATTGTAGAGAACAATTTGTC | Amplify 1 kb homologous sequence upstream *PMT6* |
| PMT-HR2-F | GAATGCTGGTCGCTATACTGGGAAGAAGGTGTAGGGCAAATATTAG | Amplify 1 kb homologous sequence downstream *PMT6* |
| PMT-HR2-R | GCAAAGCTATTAGAAAAATTCTGTAGC | Amplify 1 kb homologous sequence downstream *PMT6* |
| PMT6-F | CGTCTCTAACCAGACTGATTACTTCG | Verify *PMT6* deletion |
| PMT6-R | CAGCATTGAATATCCTCATTACCTTG | Verify *PMT6* deletion, negative control |
| Pcas-F | CAATTGACACCTTACGATTATTTAGAGAG | For constructing the plasmid pCas9-gRNA-hphB |
| Pcas-R | GGTTTAGTCCTCCTTACACCTTGTC | For constructing the plasmid pCas9-gRNA-hphB |
| phygBF | GACAAGGTGTAAGGAGGACTAAACCATGAAAAAGCCTGAACTCACCG | For constructing the plasmid pCas9-gRNA-hphB |
| phygBR | CTCTCTAAATAATCGTAAGGTGTCAATTGCTATTCCTTTGCCCTCGGAC | For constructing the plasmid pCas9-gRNA-hphB |
| seqhygcas-F | GAGCACTTCATTGTGTTGCGC | Sequencing primer of the plasmid pCas9-gRNA-hphB |
| Seqhygcas-R | GCAACGTGACACCCTGTGCAC | Sequencing primer of the plasmid pCas9-gRNA-hphB |
| Rsc1gRF | ACGAAACGAGTAAGCTCGTCACGTAGACATTATCTACCAAGTTTTAGAGCTAGAAATAGCAAGTT | For constructing *RSC1* specific Cas9-gRNA plasmid |
| Rsc1gRR | GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGACGTAGTTTGATTTGTTTAGGTAACTTGAAC | For constructing *RSC1* specific Cas9-gRNA plasmid |

| | | |
|---|---|---|
| Rvs167gRF | ACGAAACGAGTAAGCTCGTCTCAGGAAAGGATTGGTCAGAGTTTTAGAGCTAGAAATAGCAAGTT | For constructing *RVS167* specific Cas9-gRNA plasmid |
| Rvs167gRR | GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGTCAGGATTTGATTTGTTTAGGTAACTTGAAC | For constructing *RVS167* specific Cas9-gRNA plasmid |
| Pmt6gRF | ACGAAACGAGTAAGCTCGTCCCAAGGCAGTGATCACCAGAGTTTTAGAGCTAGAAATAGCAAGTT | For constructing *PMT6* specific Cas9-gRNA plasmid |
| Pmt6gRR | GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGCCAAGGTTTGATTTGTTTAGGTAACTTGAAC | For constructing *PMT6* specific Cas9-gRNA plasmid |
| rscdnF | CTCCAGAAGTTAATTCCTTCTCTGGTC | Amplify donor fragment of Rsc1$^{G22V}$ from R1-3 genomic DNA |
| rscdnR | CTCAACGTGTTCTGGGTGTTCAC | Amplify donor fragment of Rsc1$^{G22V}$ from R1-3 genomic DNA |
| rvsdnF | GAAGGTTGACCATAAGCAGGAGC | Amplify donor fragment of Rvs167$^{G9R}$ from R3-24 genomic DNA |
| rvsdnR | GTAATCTCCAACGAAGATTCCAATG | Amplify donor fragment of Rvs167$^{G9R}$ from R3-24 genomic DNA |
| pmtdnF | CGTCTCTAACCAGACTGATTACTTCG | Amplify donor fragment of Pmt6$^{V57M}$ from R5-2 genomic DNA |
| pmtdnR | CATCGGAATGAATTTGCTCTGC | Amplify donor fragment of Pmt6$^{V57M}$ from R5-2 genomic DNA |
| gRNA-F | GTGTCCTAGTTGTTATGTTTCGAGATG | Verification primer of N20 gRNA sequence |
| gRNA-R | CTTGCAACGTGACACCCTGTG | Verification primer of N20 gRNA sequence |

**Figure S1**

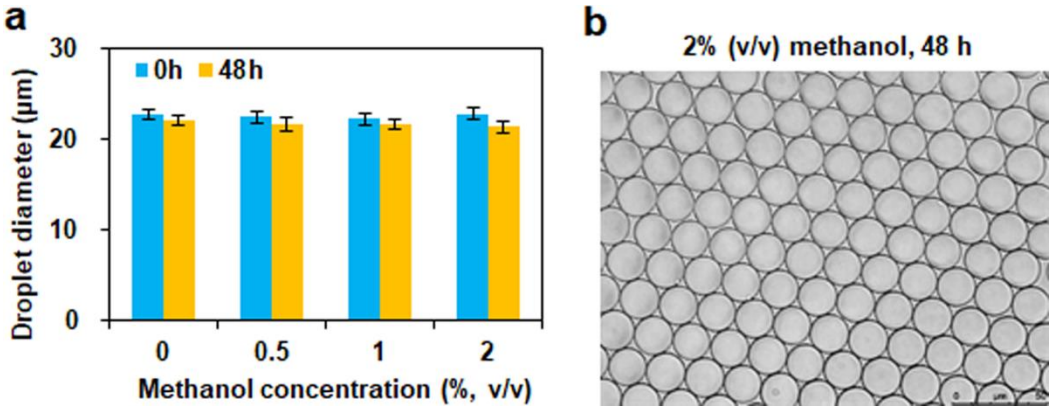


**Fig. S1 Effects of methanol concentration on droplet stability.**

**a** Mean diameter change of droplets containing different amounts of methanol. Droplets were off-chip incubated for 48 h at 30 °C. Data are presented as means ± standard deviations of three independent experiments. **b** Image of droplets containing 2% methanol at 48 h. Scale bar: 50 μm.

**Figure S2**

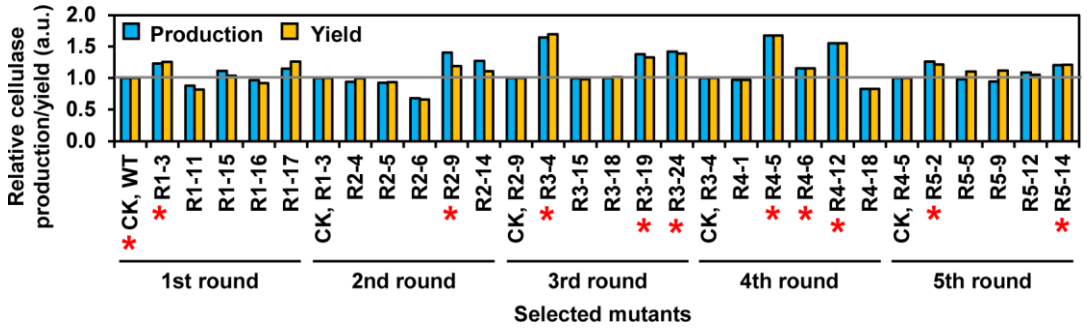


**Fig. S2 Secondary validation of selected mutants by flask fermentation.**

Top five cellulase hyperproducers from each round of five iterative ARTP mutagenesis and screening were evaluated for cellulase production by flask fermentation. Production is measured in units of cellulase activity per volume and the yield is cellulase activity per 1 $OD_{600}$ unit of cells. Data were normalized to the starting strain of each round. The verified strains were indicated by red stars, and used for the final validation and genome resequencing analysis.

**Figure S3**

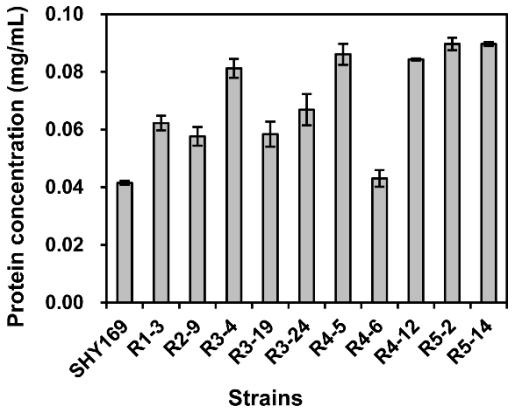


**Fig. S3 Extracellular protein concentrations of the selected mutants.**

## Figure S4

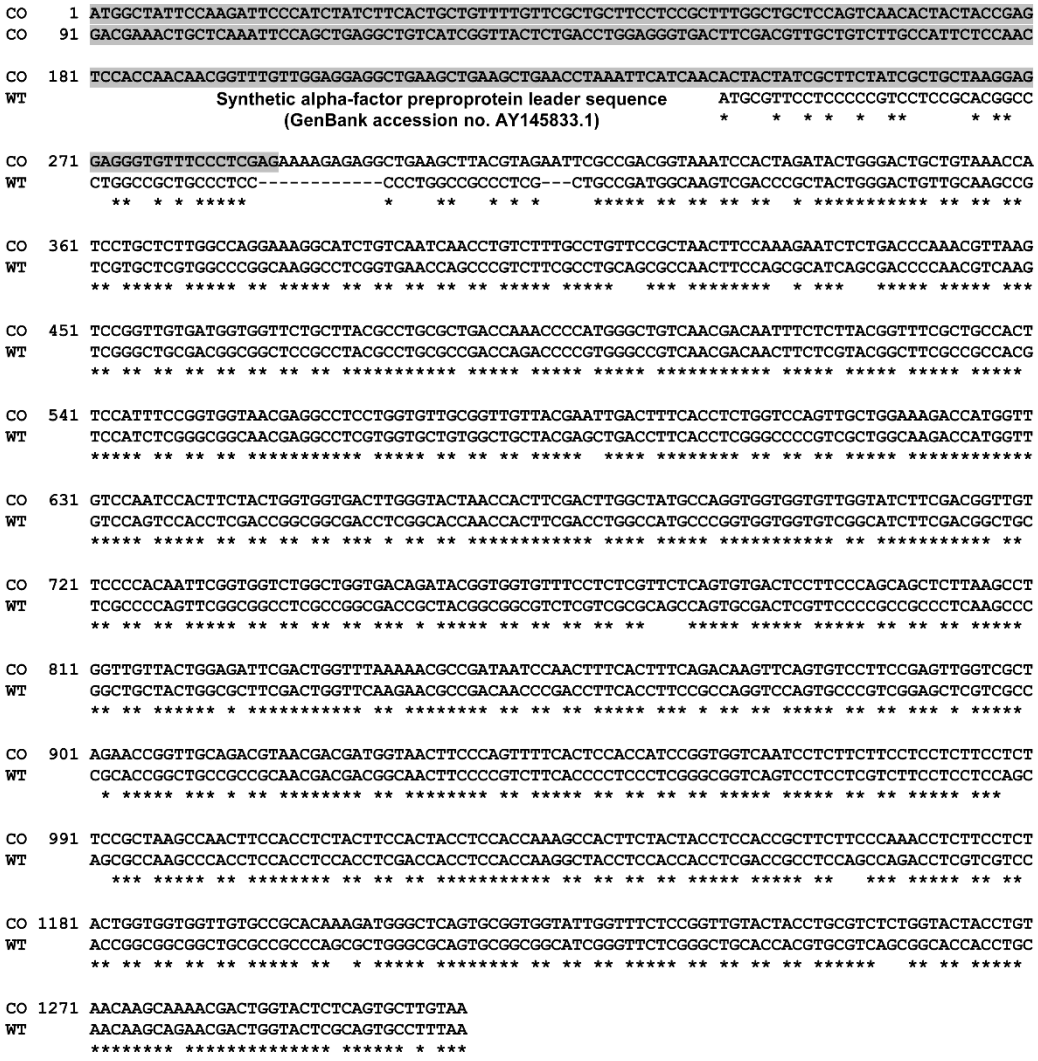


**Fig. S4 Nucleotide sequence alignment between the wild-type (WT) and codon-optimized (CO) Staphylotrichum coccosporum stce1 genes.**

The stce1 wild-type sequence was from GenBank accession no. AB248917.1.