

## Supporting Information

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

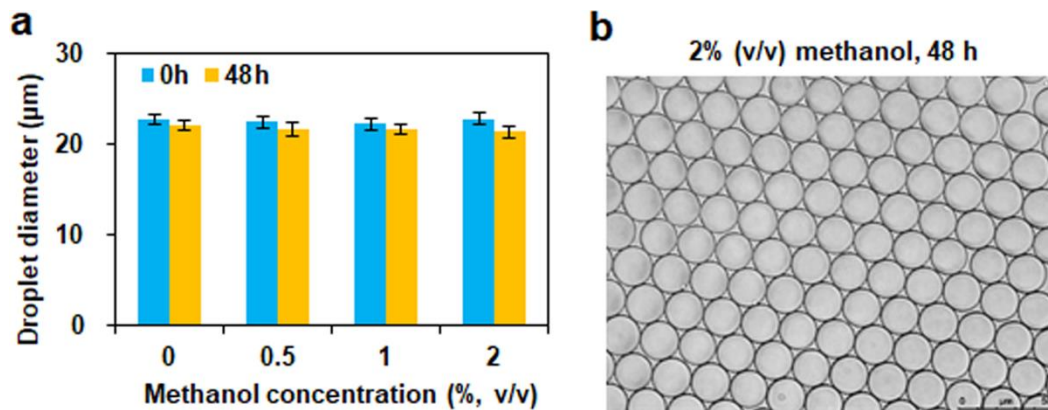
Strain	Description	Reference or source
GS115	<i>P. pastoris</i> strain, Mut <sup>+</sup>	Invitrogen, USA
SHY169	<i>P. pastoris</i> strain with integrated cellulase gene (a codon-optimized endo- $\beta$ -D-1,4-glucanase gene <i>stce1</i> from <i>Staphylotrichum coccosporum</i> ) in the genome, derived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

**Table S2. Primer used in this study**

Name	Sequence (5' → 3')	Application
HyhB-F	CACATCCGAACATAAAACAACCATGAAAAAGCCTGAACTCACCG	Amplify hphB gene
HyhB-R	CTTTTTATTGTCTAGTACTGACTATTCCTTTGCCCTCGGACG	Amplify hphB gene, reverse primer for verifying gene deletion using hphB expression cassette
Ptef-F	GACATGGAGGCCCAAGATACC	Amplify <i>S. cerevisiae</i> TEF1 promoter
Ptef-R	GGTTGTTTATGTTCCGATGTGATG	Amplify <i>S. cerevisiae</i> TEF1 promoter
Ttef-F	TCAGTACTGACAATAAAAAGATTCTTG	Amplify <i>S. cerevisiae</i> TEF1 terminator
Ttef-R	CAGTATAGCGACCAGCATTACATAC	Amplify <i>S. cerevisiae</i> TEF1 terminator
RSC-HR1-F	CATACAGATTGACTGGTGCCGATAAC	Amplify 1 kb homologous sequence upstream <i>RSC1</i>
RSC-HR1-R	GTATTCTGGGCCTCCATGTCCAGAGATCAGCGTATTTCTGTTTG	Amplify 1 kb homologous sequence upstream <i>RSC1</i>
RSC-HR2-F	GAATGCTGGTCGCTATACTGATAGGTCGATTCTGTATATTGTAACAAT	Amplify 1 kb homologous sequence downstream <i>RSC1</i>
RSC-HR2-R	AAGTTACGATACGAAGAATTGAGAAGAAG	Amplify 1 kb homologous sequence downstream <i>RSC1</i>
RSC1-F	GTCGGAGGCTTTCAAGGAAG	Verify <i>RSC1</i> deletion
RSC1-R	GATTCTTACCTTCTGTAGGGACATCG	Verify <i>RSC1</i> deletion, negative control
RVS-HR1-F	CACGCTTGATTTATGTCTGTACAAAGG	Amplify 1 kb homologous sequence upstream <i>RVS167</i>
RVS-HR1-R	GTATTCTGGGCCTCCATGTCATCGGATAGTTCGCAATTGAGG	Amplify 1 kb homologous sequence upstream <i>RVS167</i>
RVS-HR2-F	GAATGCTGGTCGCTATACTGTGCTAAATGTCAAATATCAAGAATCTATACG	Amplify 1 kb homologous sequence downstream <i>RVS167</i>
RVS-HR2-R	ACAATTAATGATCTCATGTCTGACGTG	Amplify 1 kb homologous sequence downstream <i>RVS167</i>
RVS167-F	CAGAGGAATAAGTGGCTCTGATAGTG	Verify <i>RVS167</i> deletion
RVS167-R	CTGAATCCAATTTGATGGTTCAAC	Verify <i>RVS167</i> deletion, negative control
PMT-HR1-F	CACTTGATTATCTAGGAAAGGCATTATC	Amplify 1 kb homologous sequence upstream <i>PMT6</i>
PMT-HR1-R	GTATTCTGGGCCTCCATGTCCTCGGATTGTAGAGAACAATTTGTC	Amplify 1 kb homologous sequence upstream <i>PMT6</i>
PMT-HR2-F	GAATGCTGGTCGCTATACTGGGAAGAAGGTGTAGGGCAAATATTAG	Amplify 1 kb homologous sequence downstream <i>PMT6</i>
PMT-HR2-R	GCAAAGCTATTAGAAAAATTCTGTAGC	Amplify 1 kb homologous sequence downstream <i>PMT6</i>
PMT6-F	CGTCTCTAACCAGACTGATTACTTCG	Verify <i>PMT6</i> deletion
PMT6-R	CAGCATTGAATATCCTCATTACCTTG	Verify <i>PMT6</i> 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	GAGCACTTCATTGTGTGCGC	Sequencing primer of the plasmid pCas9-gRNA-hphB
Seqhygcas-R	GCAACGTGACACCCTGTGCAC	Sequencing primer of the plasmid pCas9-gRNA-hphB
Rsc1gRF	ACGAAACGAGTAAGCTCGTCACGTAGACATTATCTACCAAGTTTTAGAGCTAGAAATAGCAAGTT	For constructing <i>RSC1</i> specific Cas9-gRNA plasmid
Rsc1gRR	GACGAGCTTACTCGTTTCGTCTCACGGACTCATCAGACGTAGTTGATTTGTTTAGTAAGTACTGAAC	For constructing <i>RSC1</i> specific Cas9-gRNA plasmid

Rvs167gRF	ACGAAACGAGTAAGCTCGTCTCAGGAAAGGATTGGTCAGAGTTTTAGAGCTAGAAATAGCAAGTT	For constructing <i>RVS167</i> specific Cas9-gRNA plasmid
Rvs167gRR	GACGAGCTTACTCGTTTCGTCTCACGGACTCATCAGTCAGGATTTGATTTGTTTAGGTAACCTGAAC	For constructing <i>RVS167</i> specific Cas9-gRNA plasmid
Pmt6gRF	ACGAAACGAGTAAGCTCGTCCCAAGGCAGTGATCACCAGATTTTAGAGCTAGAAATAGCAAGTT	For constructing <i>PMT6</i> specific Cas9-gRNA plasmid
Pmt6gRR	GACGAGCTTACTCGTTTCGTCTCACGGACTCATCAGCCAAGGTTTGATTTGTTTAGGTAACCTGAAC	For constructing <i>PMT6</i> specific Cas9-gRNA plasmid
rscdnF	CTCCAGAAGTTAATTCCTTCTCTGGTC	Amplify donor fragment of Rsc1 <sup>G22V</sup> from R1-3 genomic DNA
rscdnR	CTCAACGTGTTCTGGGTGTTTAC	Amplify donor fragment of Rsc1 <sup>G22V</sup> from R1-3 genomic DNA
rvsdnF	GAAGGTTGACCATAAGCAGGAGC	Amplify donor fragment of Rvs167 <sup>G9R</sup> from R3-24 genomic DNA
rvsdnR	GTAATCTCCAACGAAGATTCCAATG	Amplify donor fragment of Rvs167 <sup>G9R</sup> from R3-24 genomic DNA
pmt6dnF	CGTCTCTAACCAGACTGATTACTTCG	Amplify donor fragment of Pmt6 <sup>V57M</sup> from R5-2 genomic DNA
pmt6dnR	CATCGGAATGAATTTGCTCTGC	Amplify donor fragment of Pmt6 <sup>V57M</sup> from R5-2 genomic DNA
gRNA-F	GTGTCCTAGTTGTTATGTTTCGAGATG	Verification primer of N20 gRNA sequence
gRNA-R	CTTGCAACGTGACACCCTGTG	Verification primer of N20 gRNA sequence

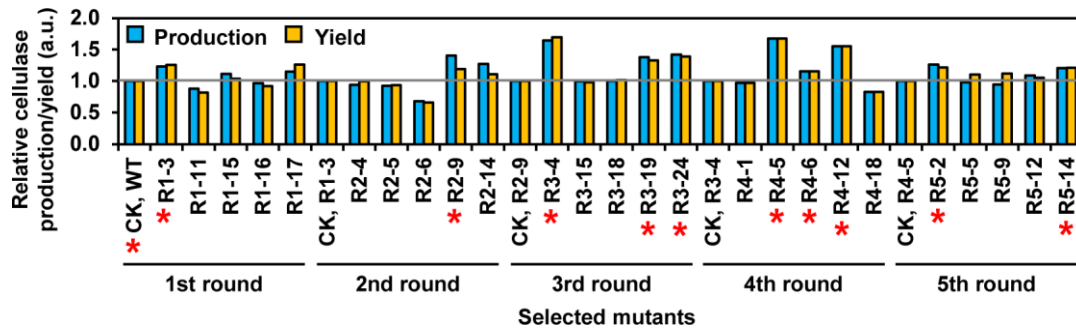
**Figure S1**



**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  $\pm$  standard deviations of three independent experiments. **b** Image of droplets containing 2% methanol at 48 h. Scale bar: 50  $\mu$ m.

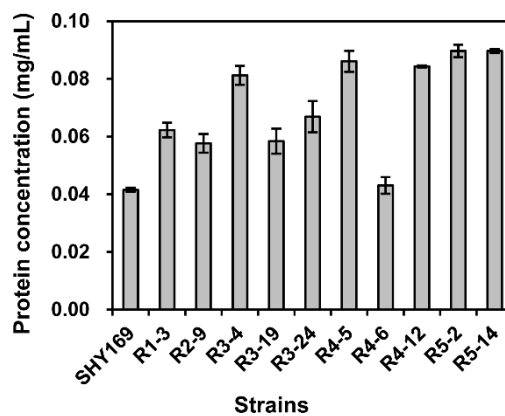
**Figure S2**



**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<sub>600</sub> 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**

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CO 1 ATGGCTATTCCAAGATTCCCATCTATCTTCACTGCTGTTTTGTTGCTGCTTCCTCCGCTTTGGCTGCCTCAGTCAACACTACTACCGAG
CO 91 GACGAAACTGCTCAAATCCAGCTGAGGCTGTCATCGGTTACTCTGACCTGGAGGGTGACTTCGACGTTGCTGTCTTGCCATTCTCCAAC

CO 181 TCCACCAACAACGGTTTGTGGAGGAGGCTGAAGCTGAAGCTGAACCTAAATTCATCAACACTACTATCGCTTCATCGCTGCTAAGGAG
WT Synthetic alpha-factor preproprotein leader sequence ATGCGTTCCTCCCCGTCTCCGCACGGCC
(GenBank accession no. AY145833.1) * * * * *

CO 271 GAGGGTGTTCCTCGAGAAAAGAGAGGCTGAAGCTTACGTAGAATTGCGCGACGGTAAATCCACTAGATACTGGGACTGTGTAAACCA
WT CTGGCCGCTGCCCTCC-----CCCTGGCCGCCCTCG---CTGCCGATGGCAAGTCGACCCGCTACTGGGACTGTGCAAGCCG
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CO 361 TCCTGCTCTTGGCCAGGAAAGCATCTGTCAATCAACCTGTCTTTCGCTGTTCGCTAACTTCCAAAGAATCTCTGACCCAAACGTTAAG
WT TCGTGTCTGTGGCCCGCAAGGCTCGGTGAACACGCCCCTCTTCGCTGCAGCCCAACTTCCAGCGCATCAGGACCCCAACGTTCAAG
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CO 451 TCCGGTGTGATGGTGGTCTGCTTACGCTGCGCTGACCAACCCCATGGGCTGTCAACGACAATTTCTCTACGGTTTCGCTGCCACT
WT TCGGCTCGCAGCCGCGCTCCGCTTACGCTGCGCCGACGACCCCGTGGCCGTCACGACAATTTCTCTACGGTTTCGCTGCCACT
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CO 541 TCCATTTCCGGTGGTAAACGAGGCTCCGCTGGTGTGCGGTTGTACGAATTGACTTTCACCTCTGGTCCAGTTGCTGGAAGACCATGGTT
WT TCCATCTCGGGCGGCAACGAGGCTCGTGGTGTGTGGCTGTACGAGCTGACCTTACCTCGGGCCCGCTCGCTGGCAAGACCATGGTT
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CO 631 GTCCAATCCACTTCTACTGGTGGTACTGGTACTAACCACCTTCGACTTGGCTATGCCAGGTGGTGGTGTGGTATCTTCGACGGTGT
WT GTCCAGTCCACTTCGACCGCGGCGACTTCGCGACCAACCACTTCGACTTGGCATGCCGCTGGGTTGGTGTGGTATCTTCGACGGTGT
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CO 721 TCCCCAATTCGGTGGTCTGGCTGGTACAGATACGGTGGTGTTCCTCTCGTTCTCAGTGTGACTCCTTCCCAGCAGCTCTTAAGCCT
WT TCGCCCCAGTTTCGGCGGCTTCGCGCGCAGCCGCTACGGCGGCTCTCGTTCGCGCAGCCAGTGGGACTCCTTCCCCGCGCCCTCAAGCC
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CO 811 GGTGTACTGGAGATTCGACTGGTTTAAAAACGCCGATAATCCAATTTCACTTTCAGACAAGTTCAGTGTCTTCCGAGTTGGTGCCT
WT GGTGTACTGGCGCTTCGACTGGTTCAAGAACCGCACAACCCGACCTTCACTTCCGCCAGGTCCAGTGCCTGCGGCTCGGAGCTCGTGC
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CO 901 AGAACCGTTGCGAGCGTAACGACGATGGTAACTTCCAGTTTCACTCCACCATCCGGTGGTCAATCTCTTCTTCTCTCTCTCTCT
WT CGCACCGGTGCGCGCAACGACGACGGCAACTTCCCGCTTCTACCCCTCCCTCGGGCGGTGAGTCTCTCTCTCTCTCTCTCTCTCT
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CO 991 TCCGCTAAGCAACTTCCACTTACTTCCACTACCTCCACCAAGCCACTTCTACTACCTCCACCGCTTCTTCCCAAACCTCTTCTCT
WT AGCGCCAAGCCACTTCCACTTCCACTCGACCACCTCCACCAAGGCTACTTCCACCCTCGACCCTCCAGCCAGACCTCGTCTGCT
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CO 1181 ACTGGTGGTGGTGTGCGGCACAAGATGGGCTCAGTGCGGTGGTATTGGTTTCTCCGGTTGACTACCTGCGTCTCTGGTACTACCTGT
WT ACCGGCGGCGGCTGCGCCCGCAGCGCTGGGCGCAGTGCGGCGCATCGGGTCTCGGGCTGCACCACGTGCGTCAAGCGGACCACTG
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CO 1271 AACAAAGCAAAACGACTGGTACTCTCAGTGTGTTAA
WT AACAAAGCAAAACGACTGGTACTCTCAGTGTGTTAA
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**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.