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Supplementary Materials for

Evolutionary engineering of *Saccharomyces cerevisiae*: Crafting a synthetic methylotroph via self-reprogramming

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The PDF file includes:

Figs. S1 to S27
Tables S1 to S5
Legends for data S1 and S2

Other Supplementary Material for this manuscript includes the following:

Data S1 and S2

Supplementary Figures

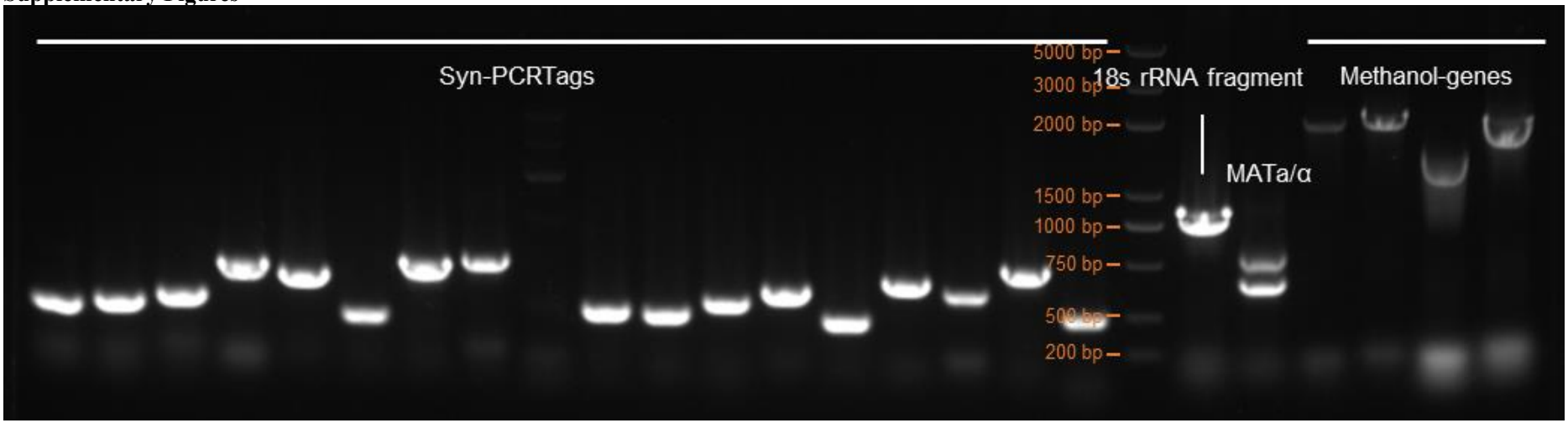


Fig. S1. PCRTag analysis for diploids demonstrates that SCDM001 contained both the synthetic chromosome and the methanol assimilation pathway.

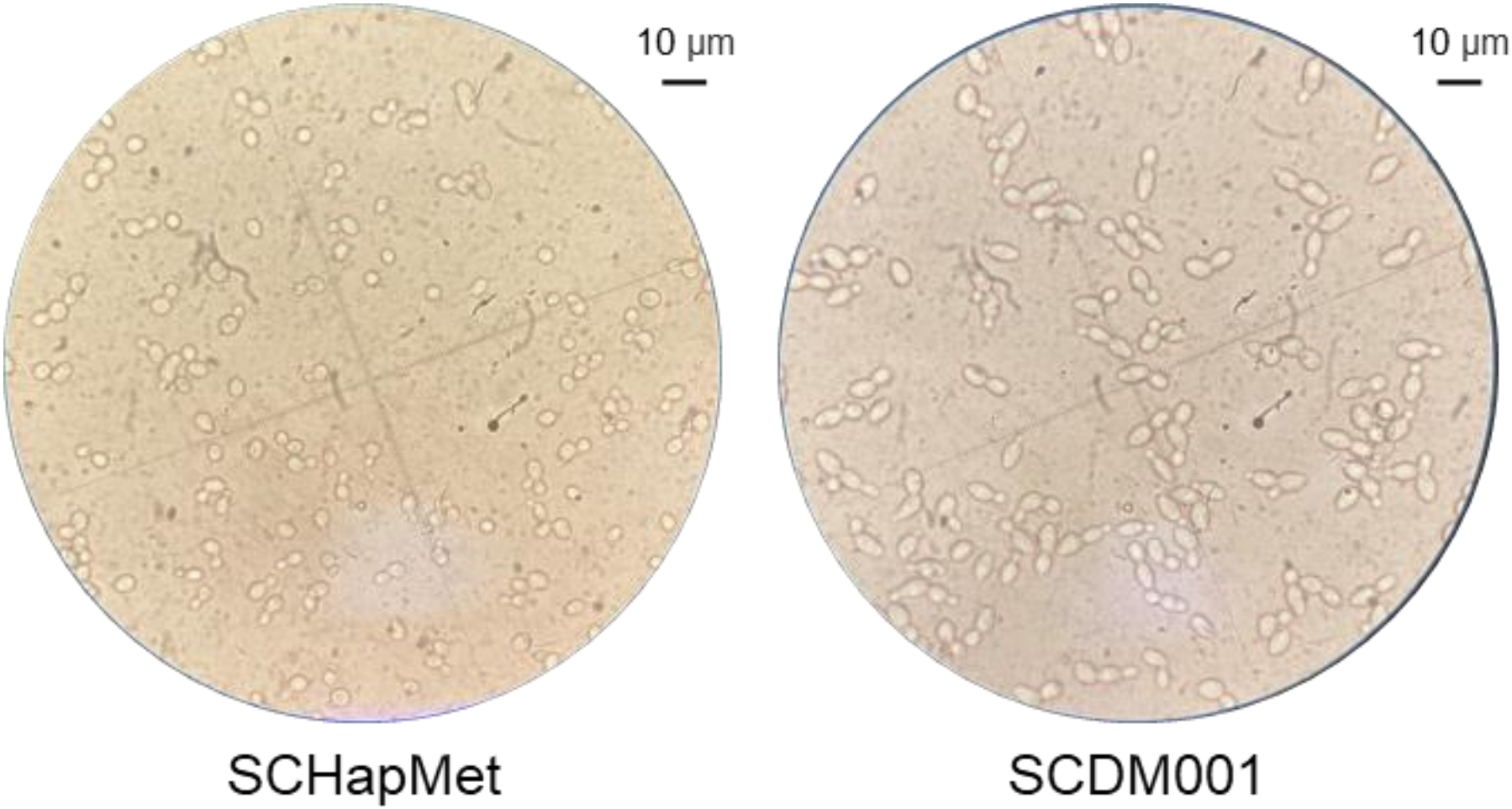


Fig. S2. Comparison of cell morphology of haploid SCHapMet and diploid SCDM001.

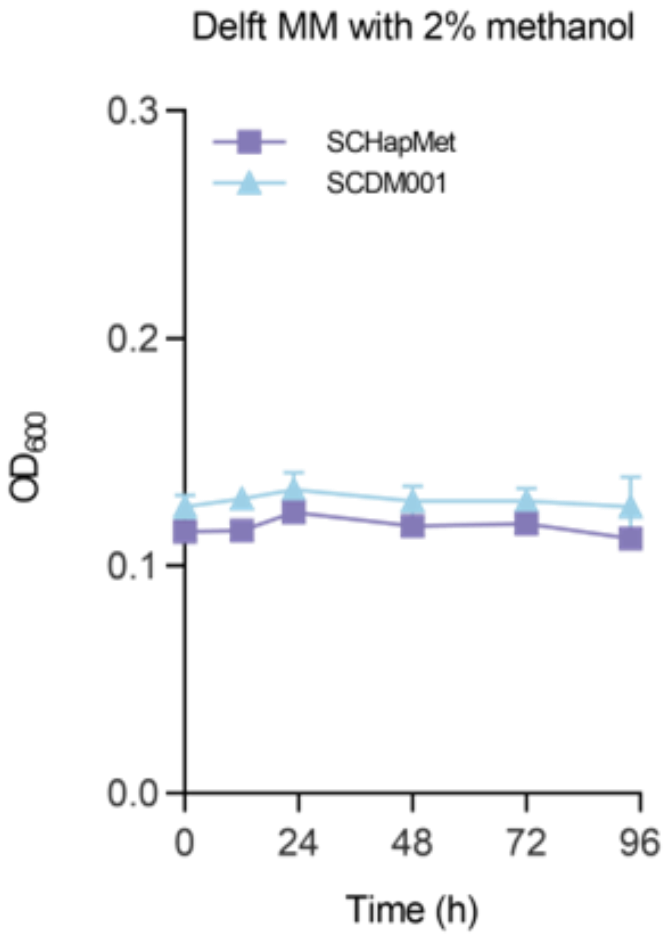


Fig. S3. Growth curves of the haploid methanol-utilizing strain SCHapMet and diploid methanol-utilizing strain SCDM001 in Delft MM with 2% methanol. All data are presented as mean ± s.d. Triplicate independent samples were adopted for each panel.

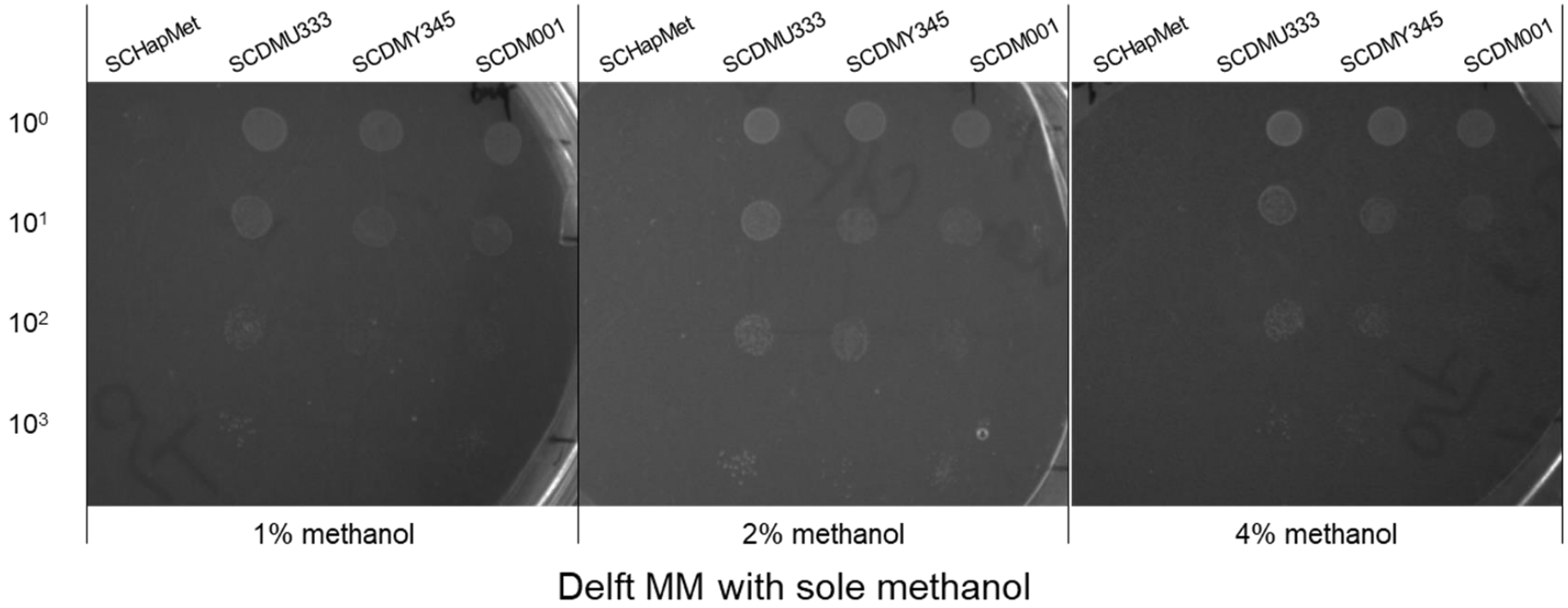


Fig. S4. Serial dilution assay comparing the growth of the SCRaMbLEd strain on Delft MM with 1%, 2%, and 4% methanol.

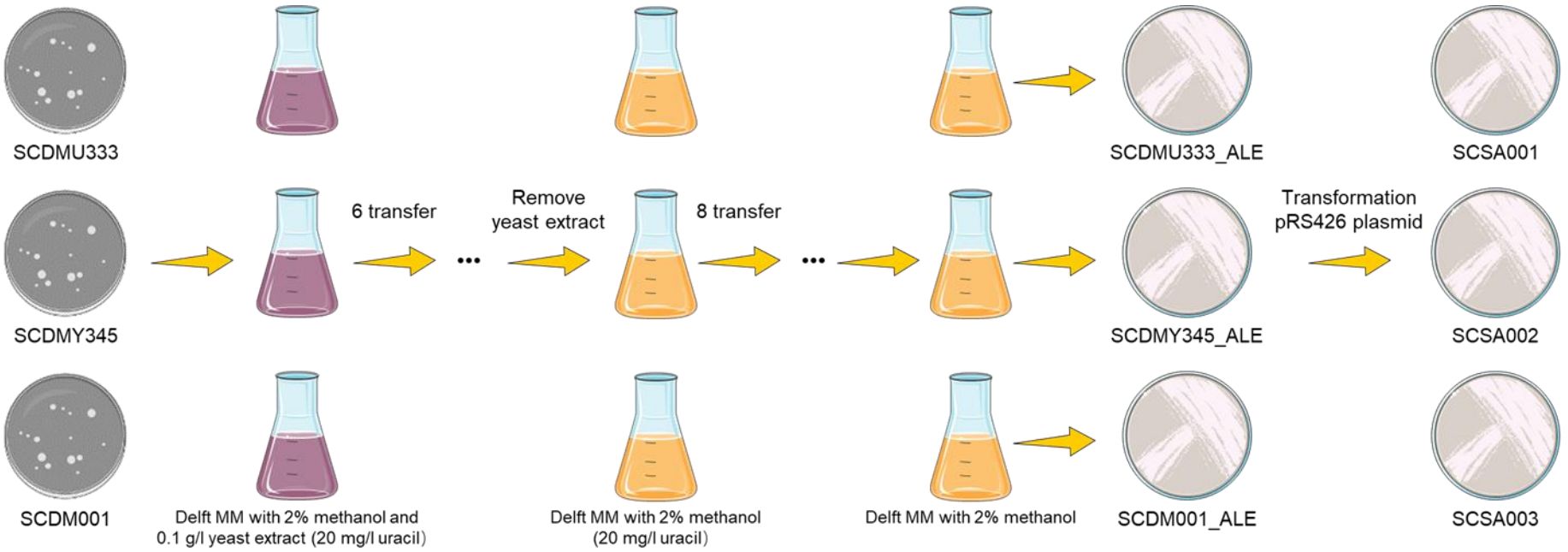


Fig. S5. Workflow for ALE of strain SCDMU333, SCDMY345, and SCDM001. Strains were cultivated in Delft MM with 2% methanol and 0.1 g/l yeast extract (20 mg/l uracil was supplemented to compensate for uracil auxotroph). After 7 passages, the strains were transferred to Delft MM with 2% methanol (20 mg/l uracil was supplemented to compensate for uracil auxotroph, removing the addition of yeast extract). If the OD_{600} achieved up to 0.4 or a single culture reached 7 days, the evolved strains were transferred to a new medium. until the strains could reach a stable growth rate in the Delft MM containing 2% sole methanol. Finally, the empty pRS426 plasmid was transformed into the evolved strains, generating strain SCSA001, SCSA002, and SCSA003.

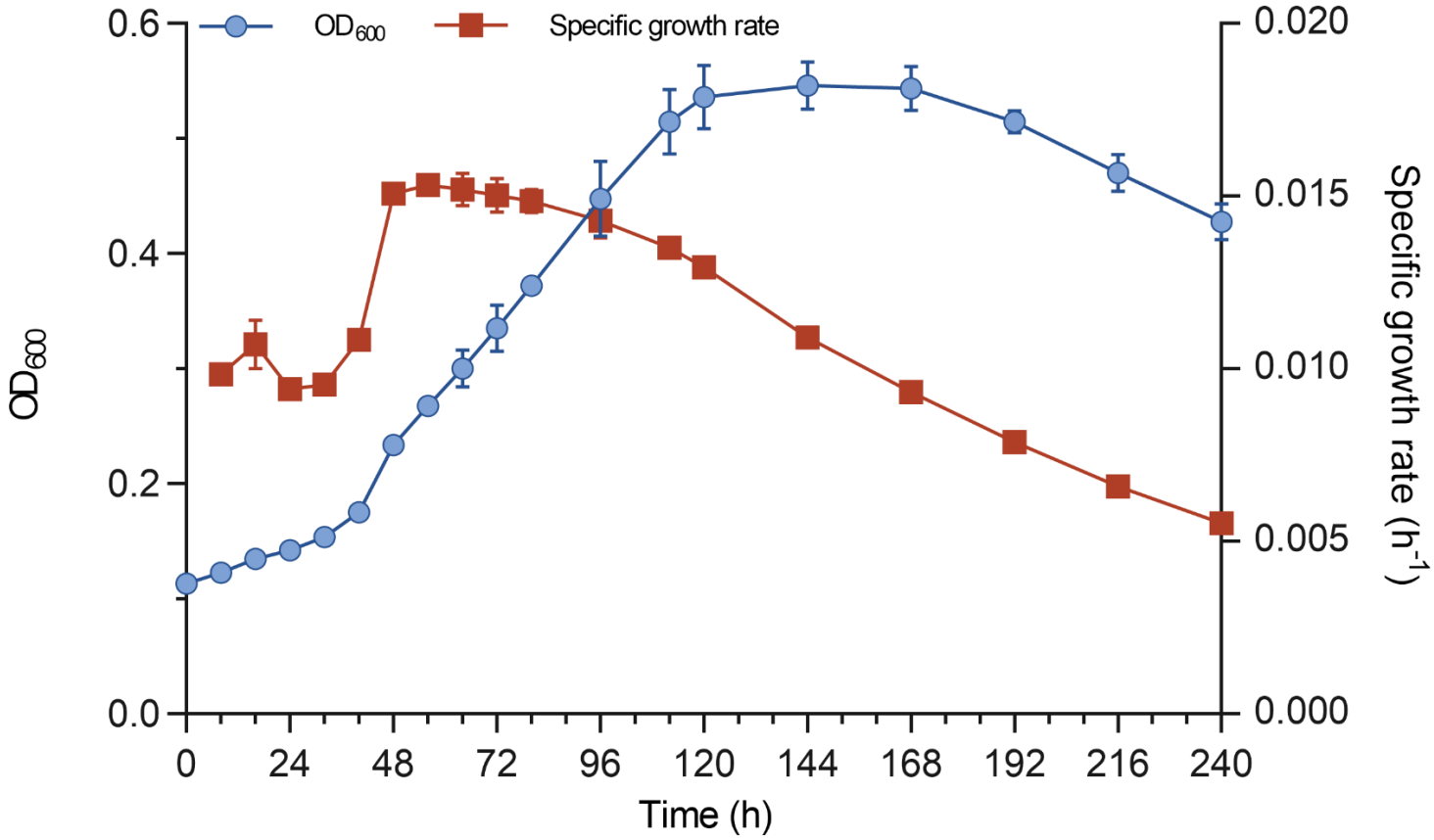


Fig. S6. Growth curves and specific growth rate of strain SCSA001 in Delft MM containing 2% methanol. The specific growth rate at any given point is calculated based on the growth from 0 hours to that time. All strains were precultured in YPD medium and washed twice using PBS (pH = 7.2) before cultivated in Delft MM. All data represent the mean ± s.d. (n = 3 biologically independent samples).

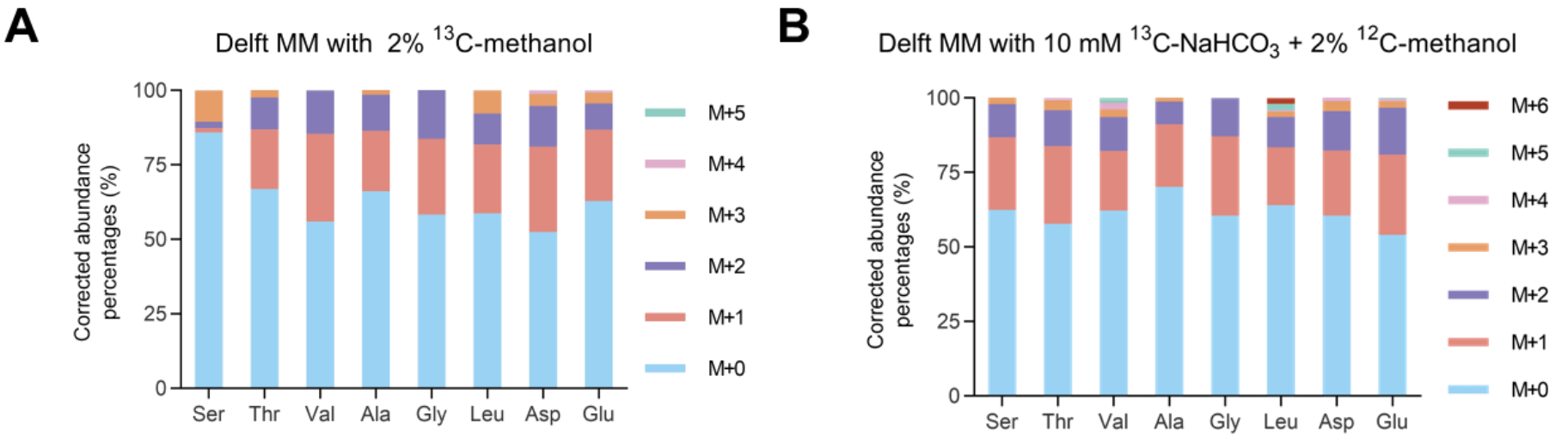


Fig. S7. Labelling pattern of amino acids in SCSA001 strain in Delft MM with 2% ^{13}C-methanol. (A) and 10 mM ^{13}C-$NaHCO_3$ + 2% ^{12}C-methanol (B); M + N, where M represents the nominal mass of the unlabeled metabolite and N indicates the total ^{13}C-labelled carbon numbers in the metabolite. Strain SCSA001 was precultured in Delft MM with ^{13}C-labelled methanol three times before being cultured in Delft MM with ^{13}C-labelled methanol. All data are presented as mean. Triplicate independent samples were adopted for each panel.

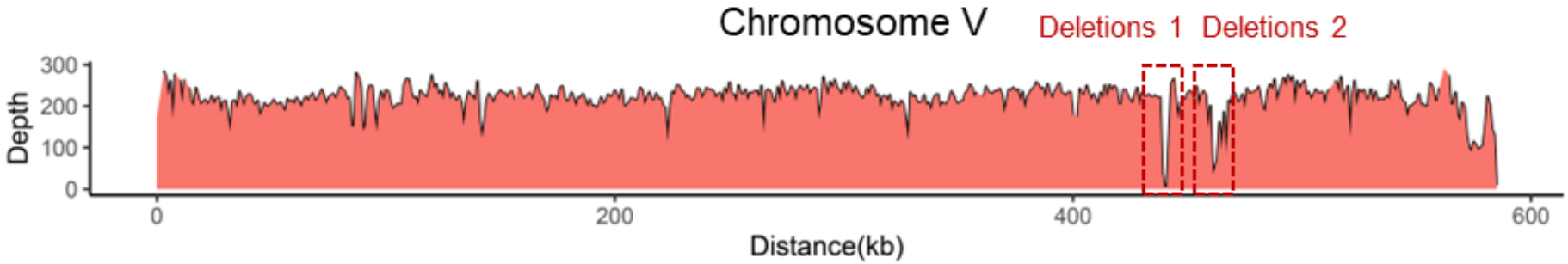
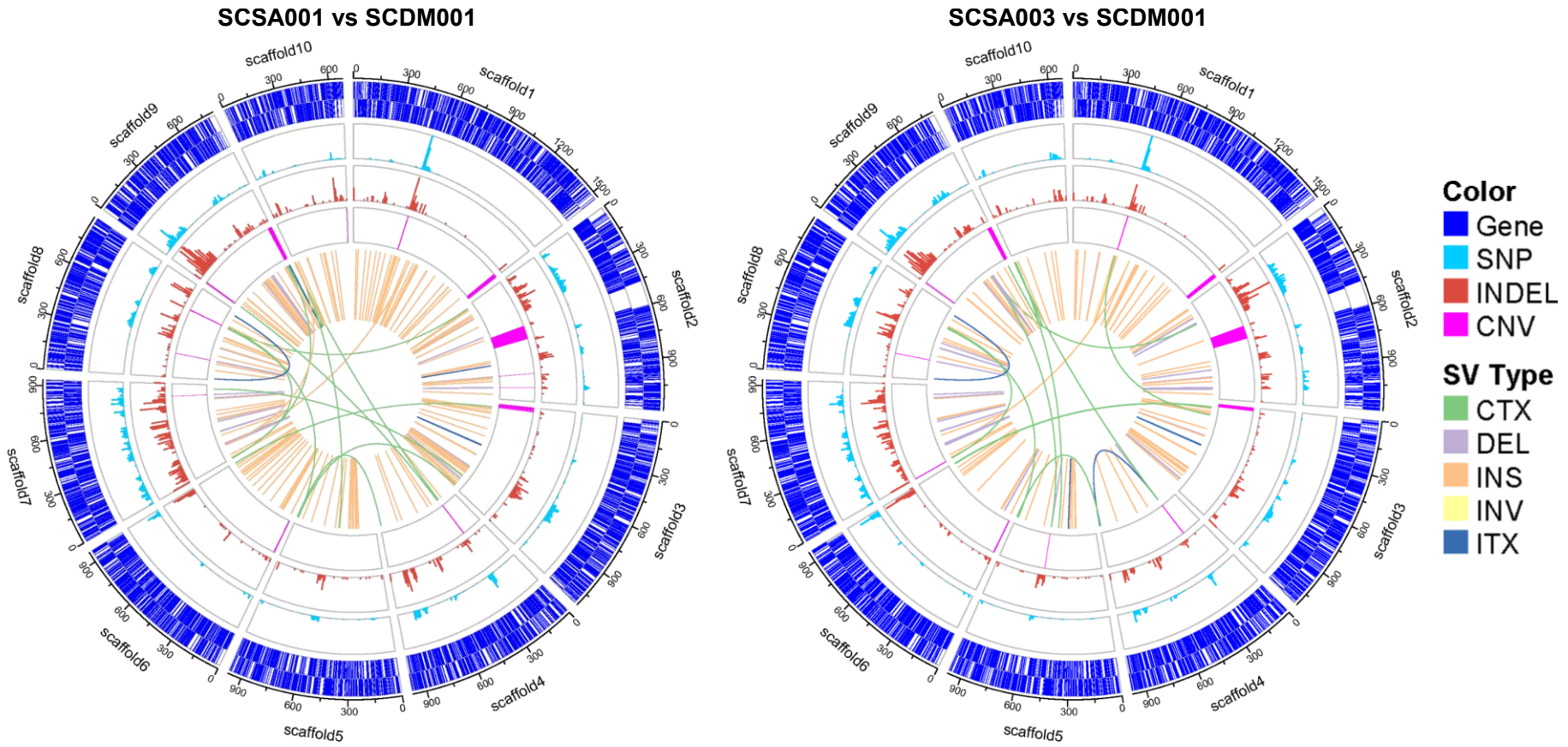


Fig. S8. Average sequencing depth per segment along synV of SCSA001. Two deletions are highlighted.



Mutation type	Exonic SNP	InDel		CNV		SV				
		Insertion	Deletion	deletion	duplication	Inter-chromosomal translocation	Deletion	Insertion	Inversion	Intra-chromosomal translocation
SCSA001	12,796	903	1,343	14	10	15	47	187	6	12
SCSA003	12,315	961	1,375	11	6	17	55	108	4	9

Fig. S9. Whole genome sequencing statistics of strains SCSA003 and SCSA001. Strain SCDM001 was used as the reference genome for comparative analysis.

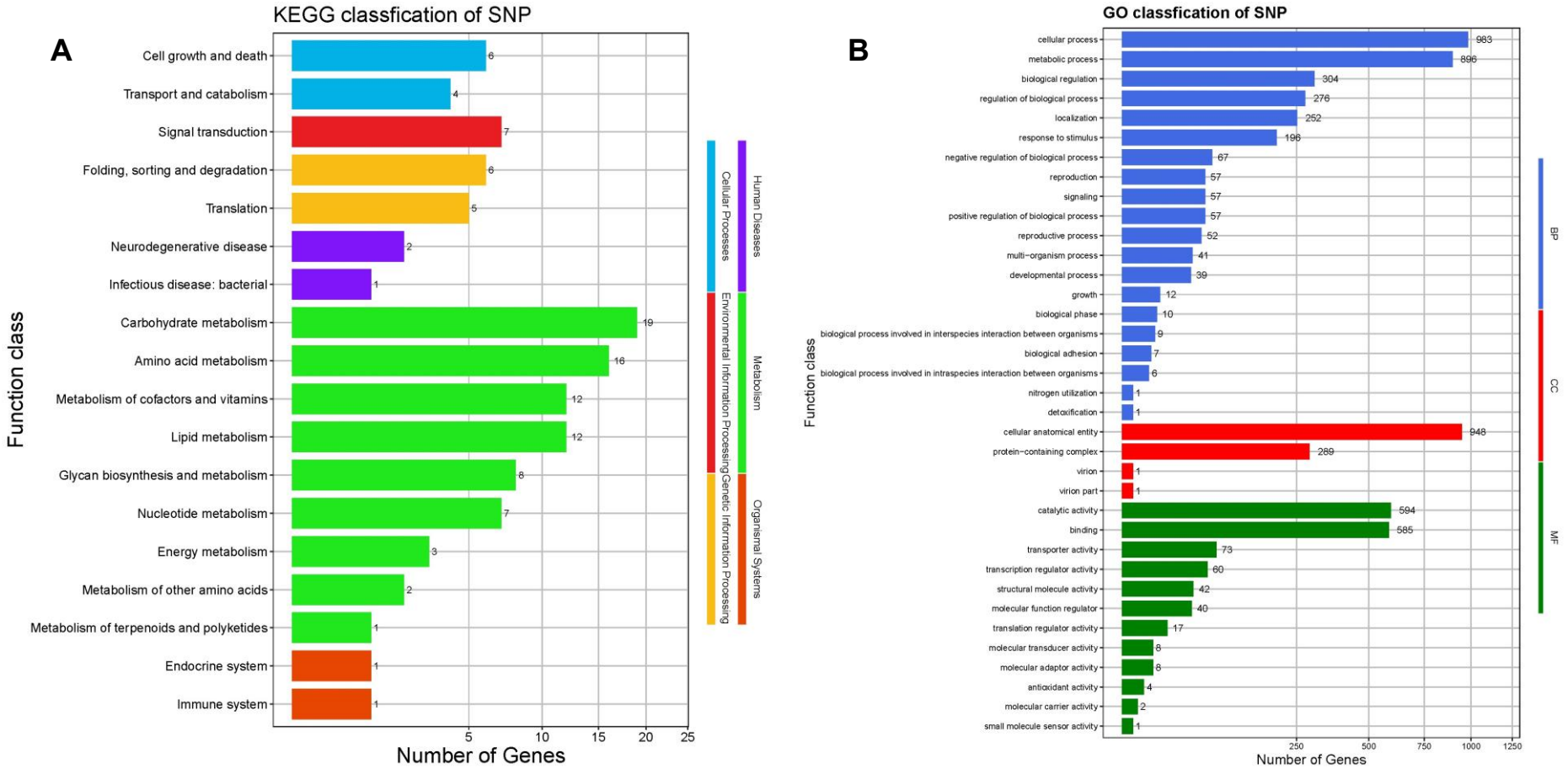


Fig. S10. KEGG pathways and GO enrichment analysis of SNPs in strain SCSA001. (A) KEGG classification of SNPs; (B) GO classification of SNPs.

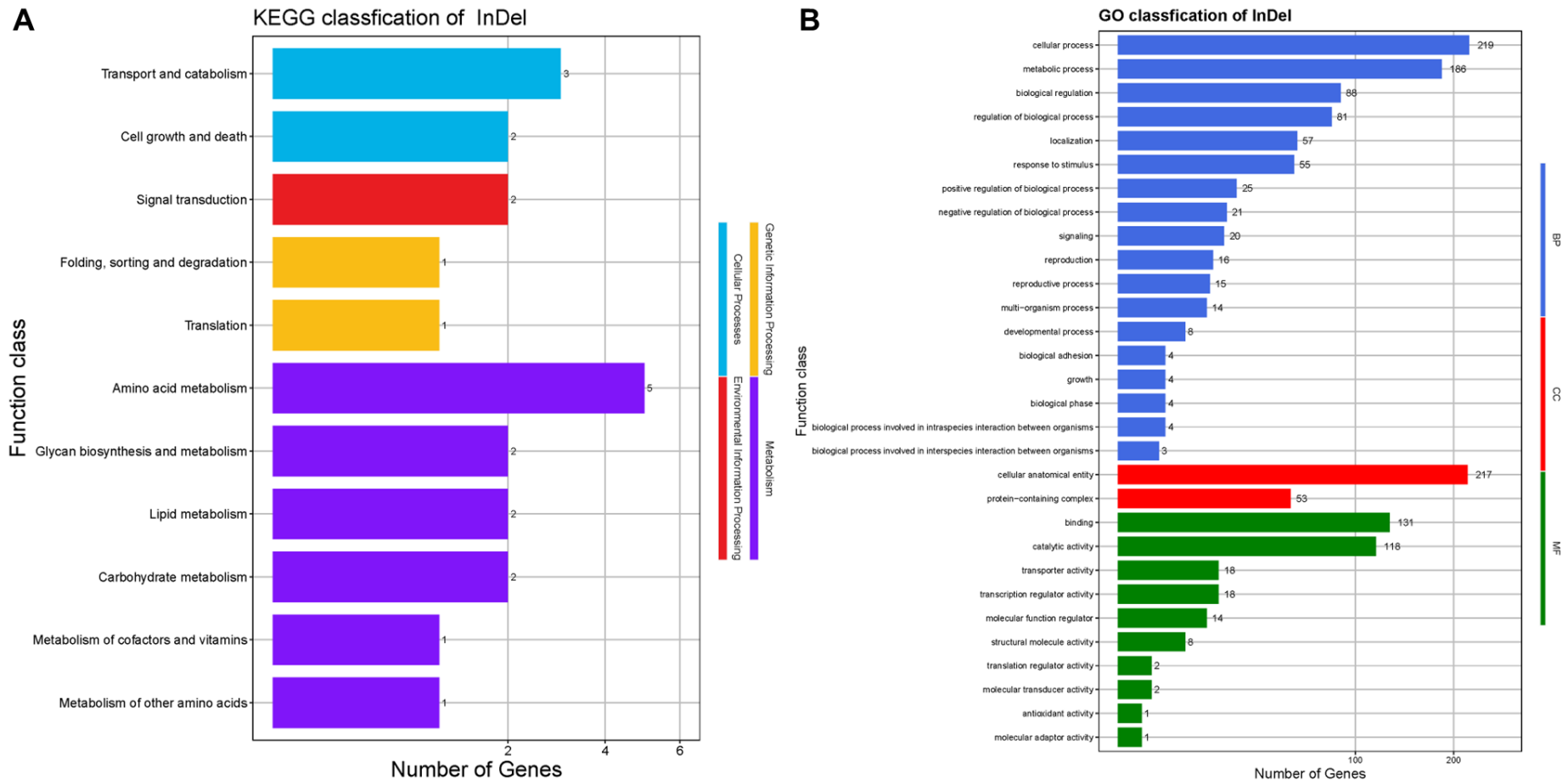


Fig. S11. KEGG pathways and GO enrichment analysis of InDels in strain SCSA001. (A) KEGG classification of InDels; (B) GO classification of InDels.

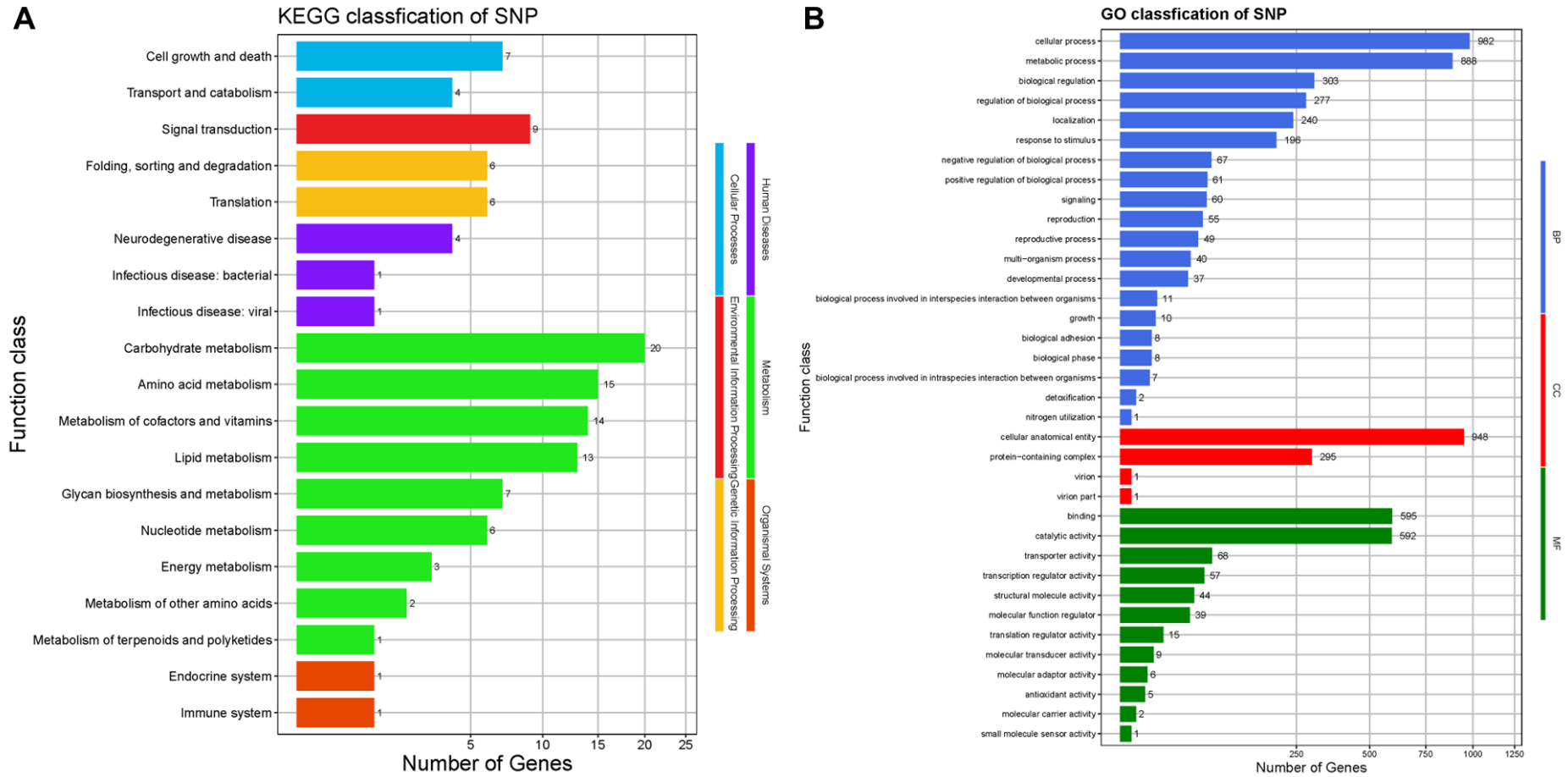


Fig. S12. KEGG pathways and GO enrichment analysis of SNPs in strain SCSA003. (A) KEGG classification of SNPs; (B) GO classification of SNPs.

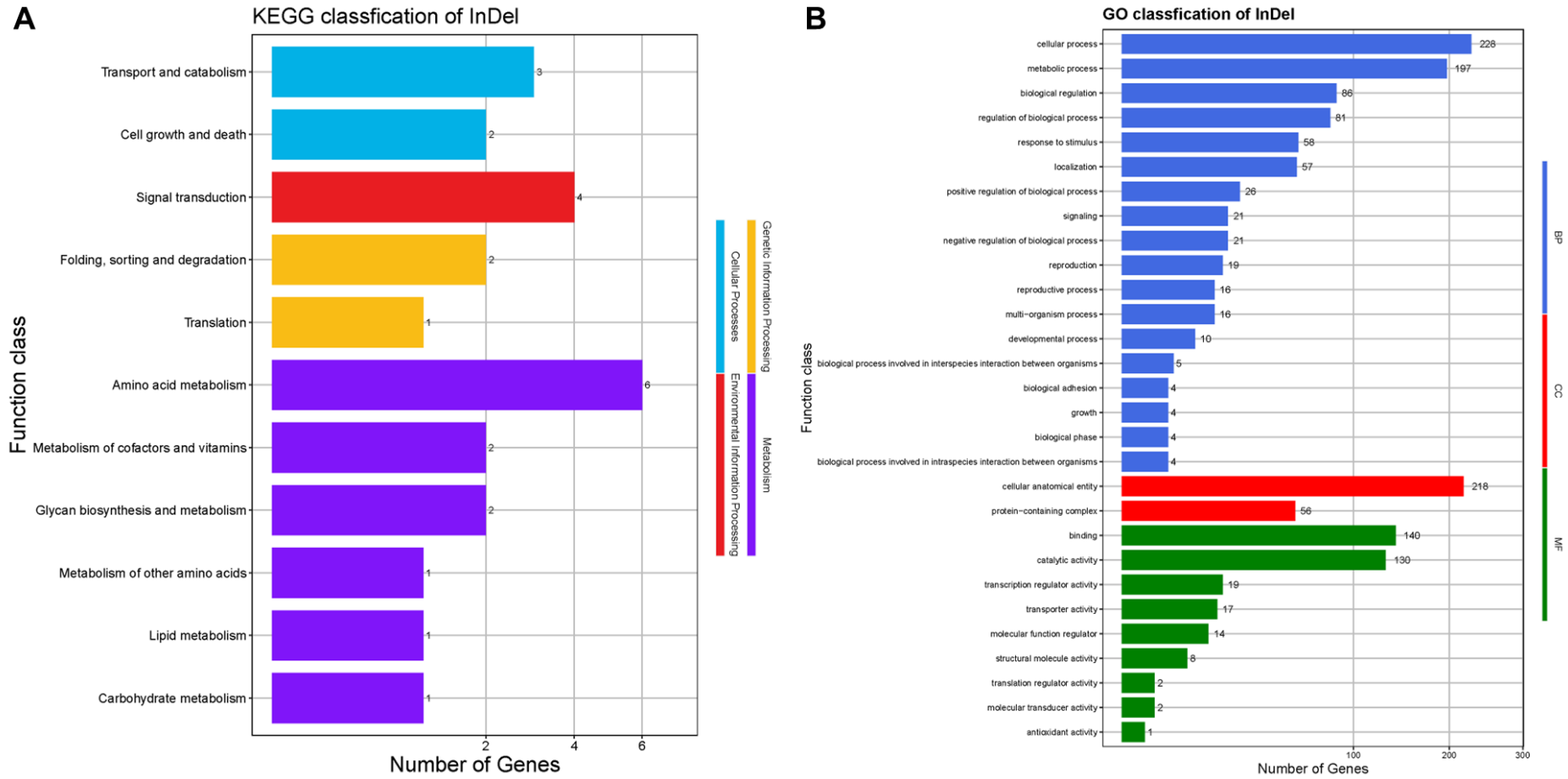


Fig. S13. KEGG pathways and GO enrichment analysis of InDels in strain SCSA003. (A) KEGG classification of InDels; (B) GO classification of InDels.

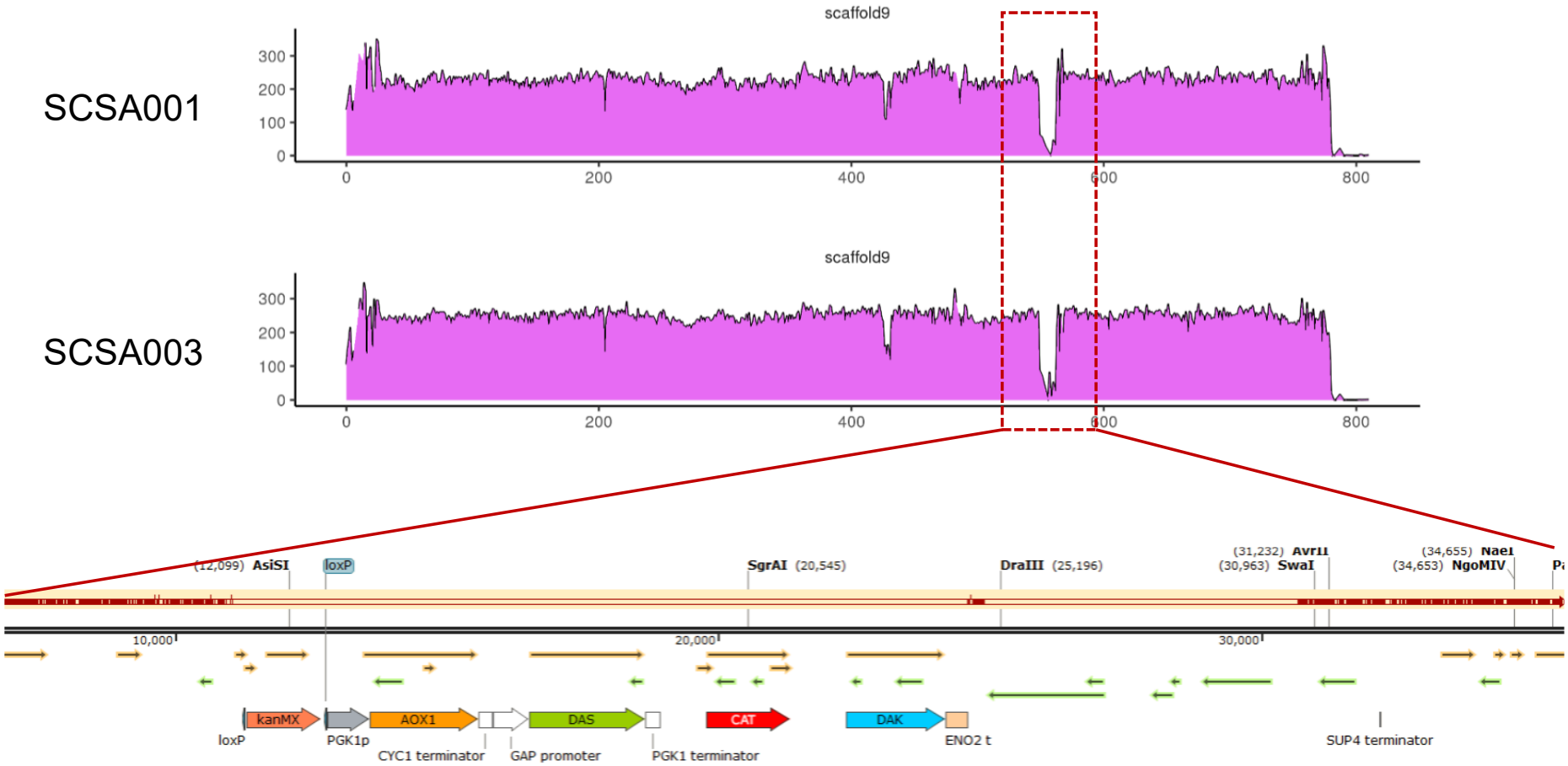


Fig. S14. Whole-genome sequencing reveals the complete eradication of the *P. pastoris*-derived methanol assimilation module in ALE-adapted strains SCSA001 and SCSA003.

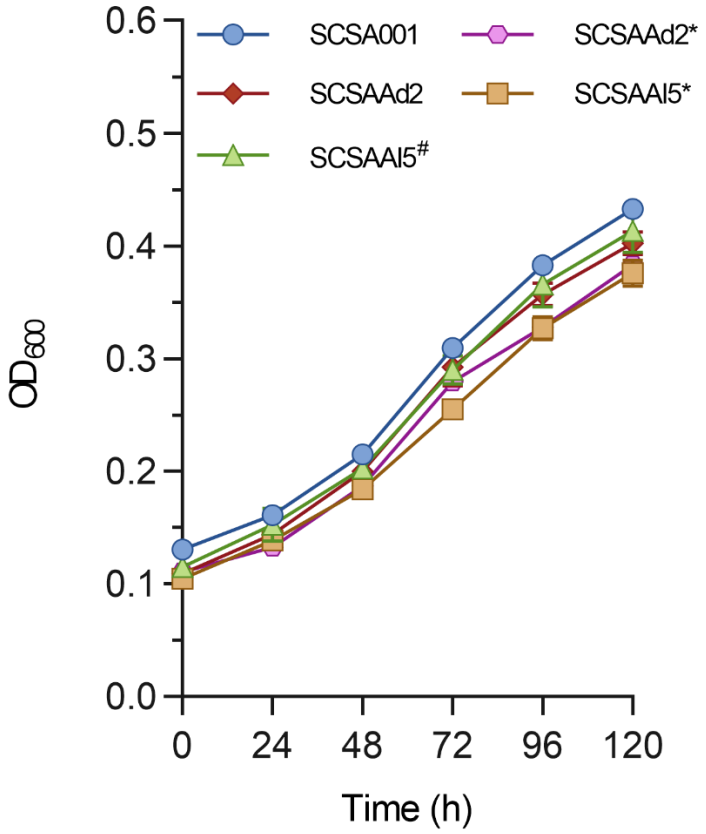


Fig. S15. Growth curves of strain SCSA001, SCSAAd2, SCSAAl5$^{\#}$, SCSAAd2* and SCSAAl5* in Delft MM with 2% methanol. All strains were precultured in YPD medium and washed twice using PBS (pH = 7.2) before cultivated in Delft MM. All data represent the mean ± s.d. (n = 3 biologically independent samples).

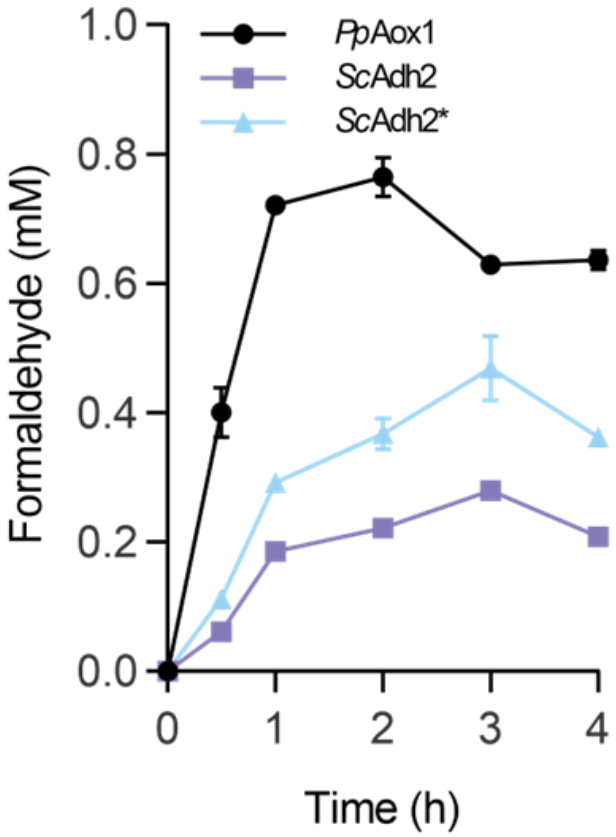


Fig. S16. Measurement of *in vitro* activities of Adh2, Adh2* and *Pp*Aox1 enzymes. All data are presented as mean ± s.d. Triplicate independent samples were adopted for each panel.

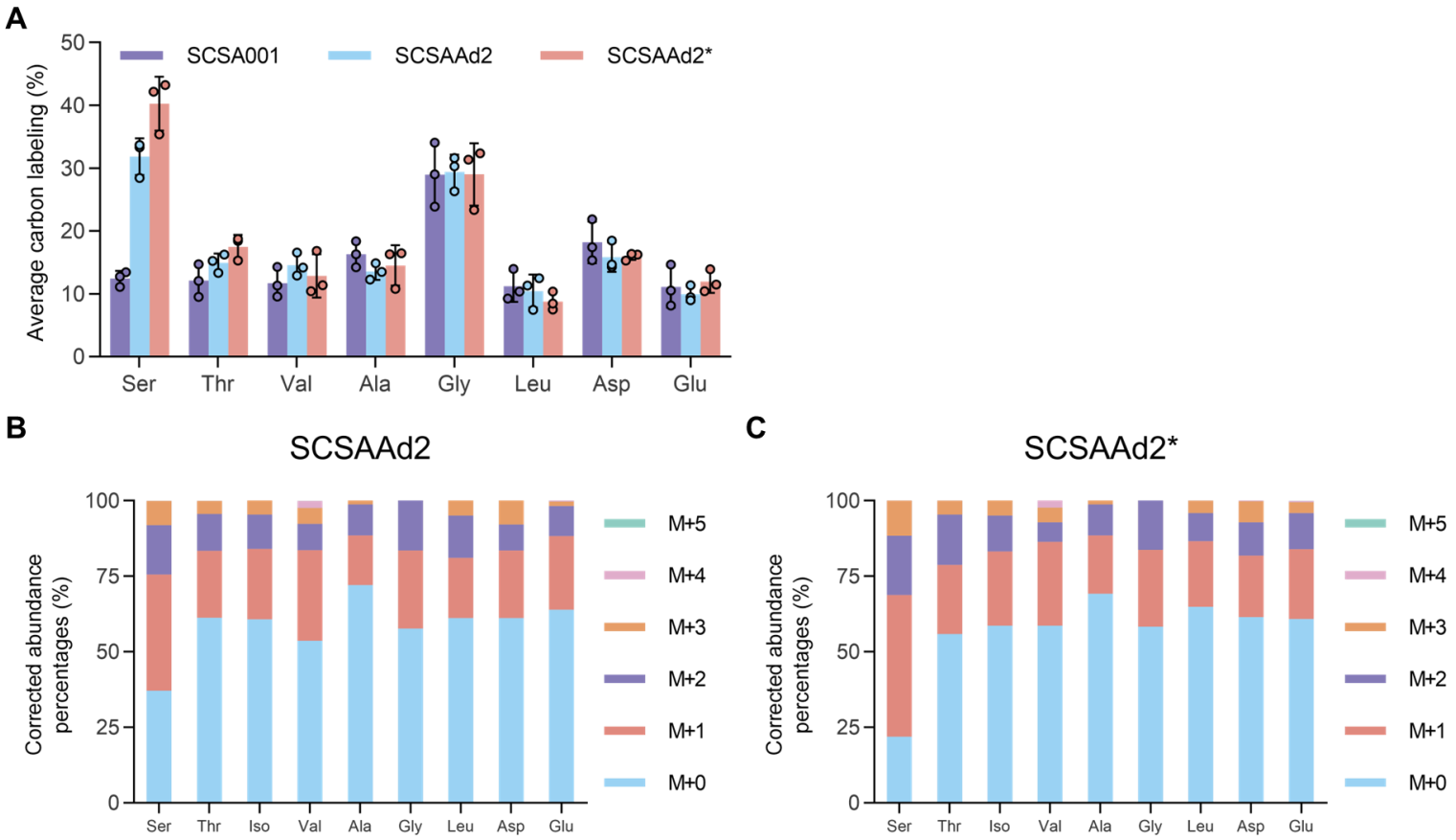


Fig. S17. Effects of Adh2 and Adh2* expression on amino acid synthesis in evolved strain SCSA001. (A) The average carbon labeling of amino acids of SCSA001, SCSAAd2, and SCSAAd2* in Delft MM supplemented with ^{13}C-labelled methanol. Strains were precultured in Delft MM with ^{13}C-labelled methanol three times before being cultured for *in vivo* ^{13}C-methanol metabolic tracer assays. (B and C) Labelling pattern of amino acids in SCSAAd2 and SCSAAd2*; M + N, where M represents the nominal mass of the unlabeled metabolite and n indicates the total 13C-labelled carbon numbers in the metabolite. Strain SCSAAd2 and SCSAAd2* were precultured in Delft MM with 13C-labelled methanol three times before being cultured in Delft MM with 13C-labelled methanol. All data are presented as mean. Triplicate independent samples were adopted for each panel.

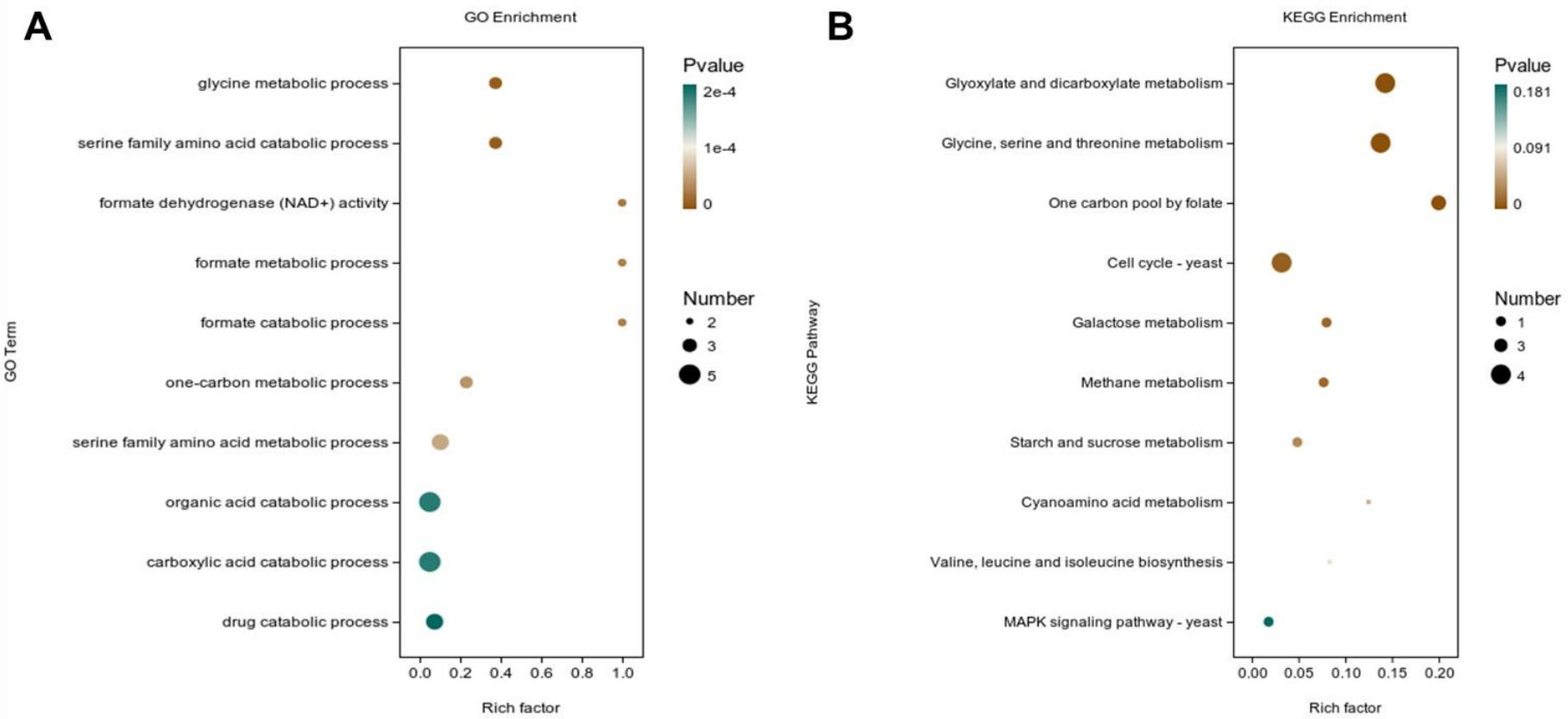


Fig. S18. GO and KEGG pathway enrichment analysis of the different expressed genes. (A) GO pathway enrichment analysis; (B) KEGG pathway enrichment analysis.

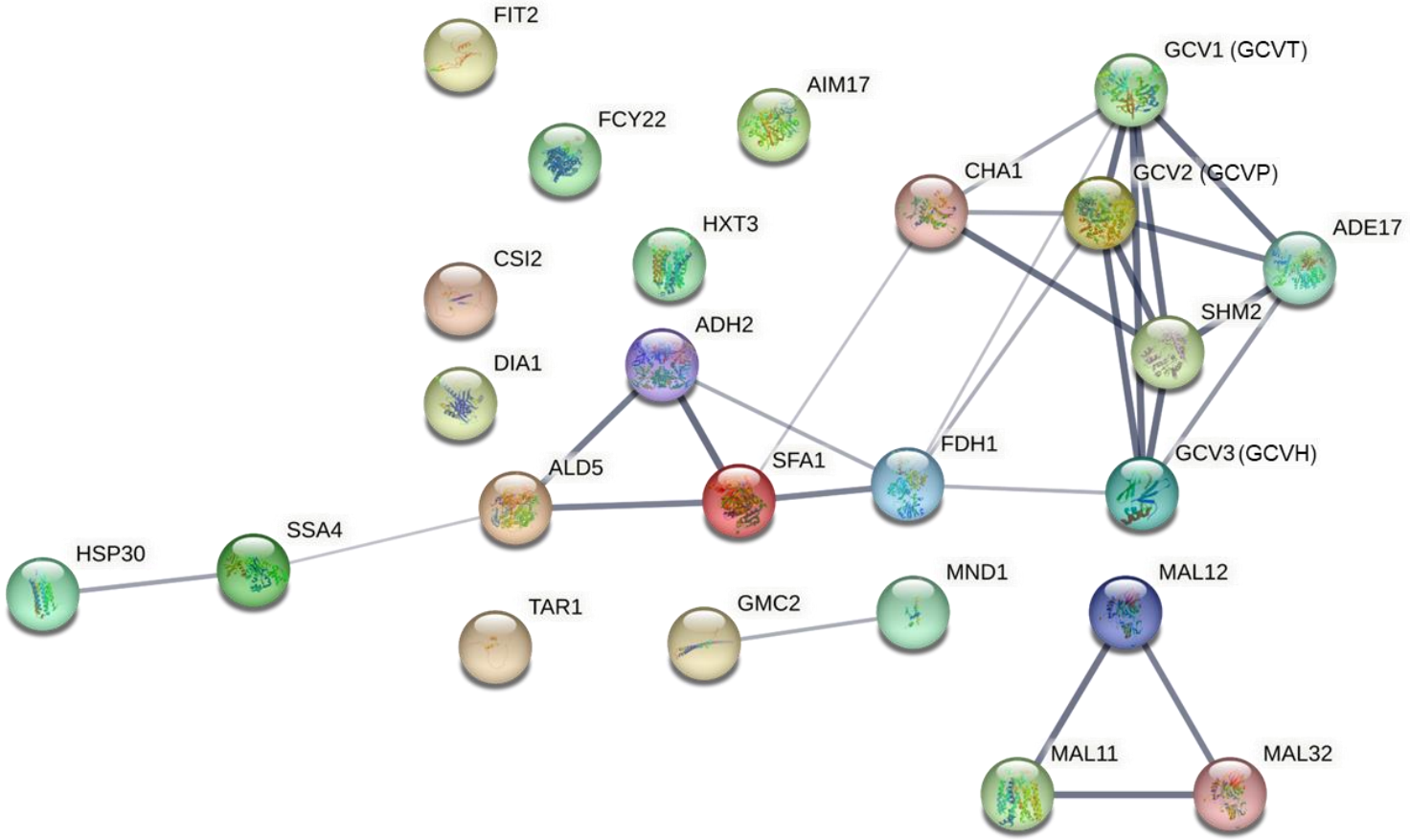


Fig. S19. The protein-protein interaction (PPI) network for the 22 differentially expressed genes together with Adh2 and Ald5. The STRING database was used to generate PPIs in DEGs with the STRING tool (Version: 12.0), based on interactions with a medium combined score > 0.4. Line thickness indicates the strength of data support.

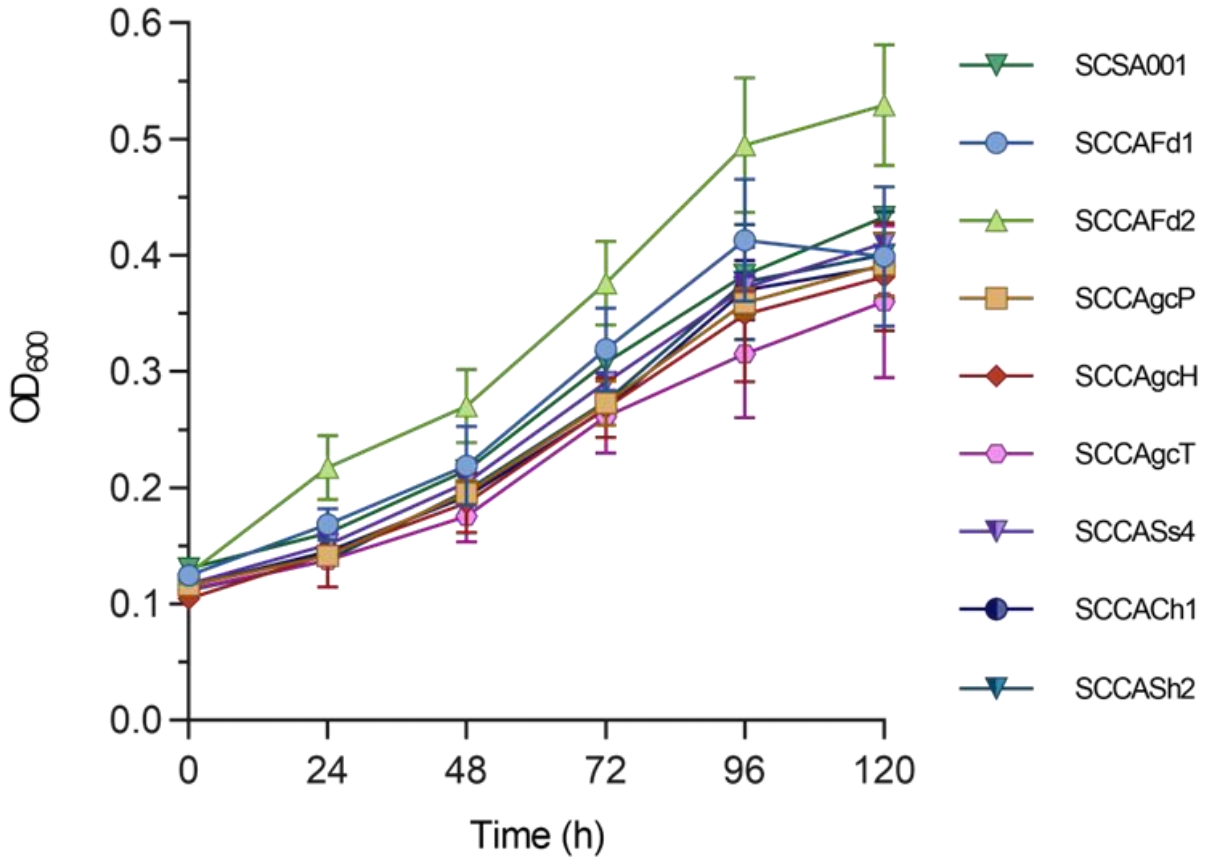


Fig. S20. Growth curves of strain SCSA001, SCSAFd1, SCSAFd2, SCSAgcP, SCSAgcH, SCSAgcT, SCSASs4, SCSACh1 and SCSASh2 in Delft MM with 2% methanol. All strains were precultured in YPD medium and washed twice using PBS (pH = 7.2) before cultivated in Delft MM. All data represent the mean ± s.d. (n = 3 biologically independent samples).

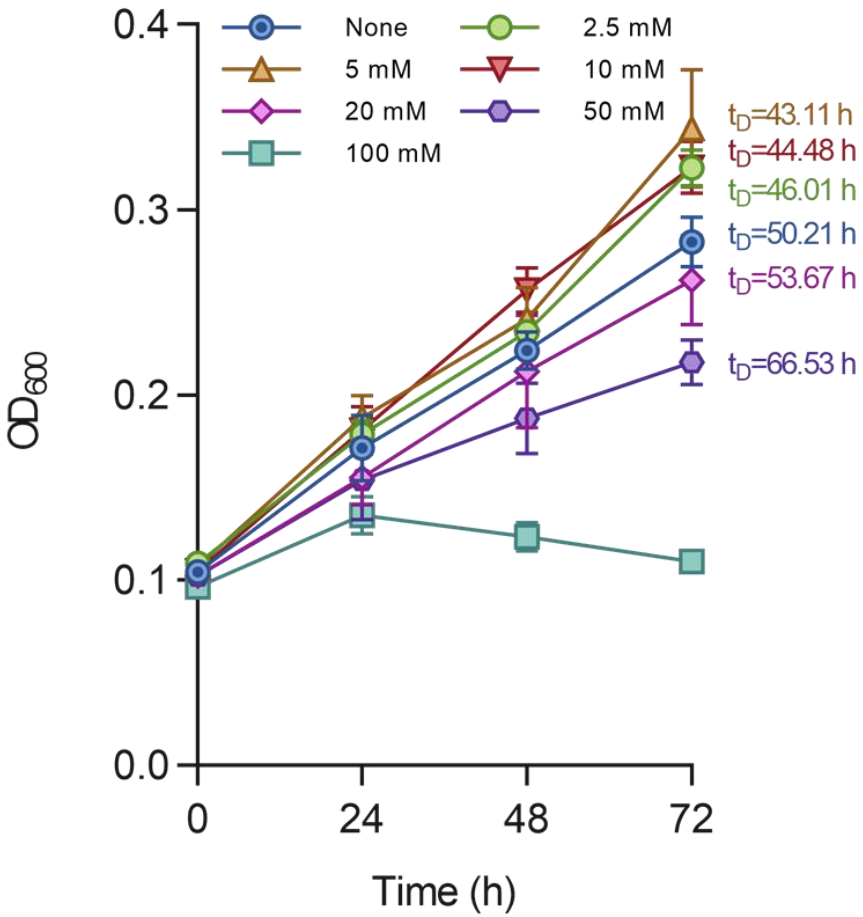


Fig. S21. Growth curves of strain SCSA001 on Delft MM containing 2% methanol and different concentrations of $NaHCO_3$. All data are presented as mean ± s.d. Triplicate independent samples were adopted for each panel.

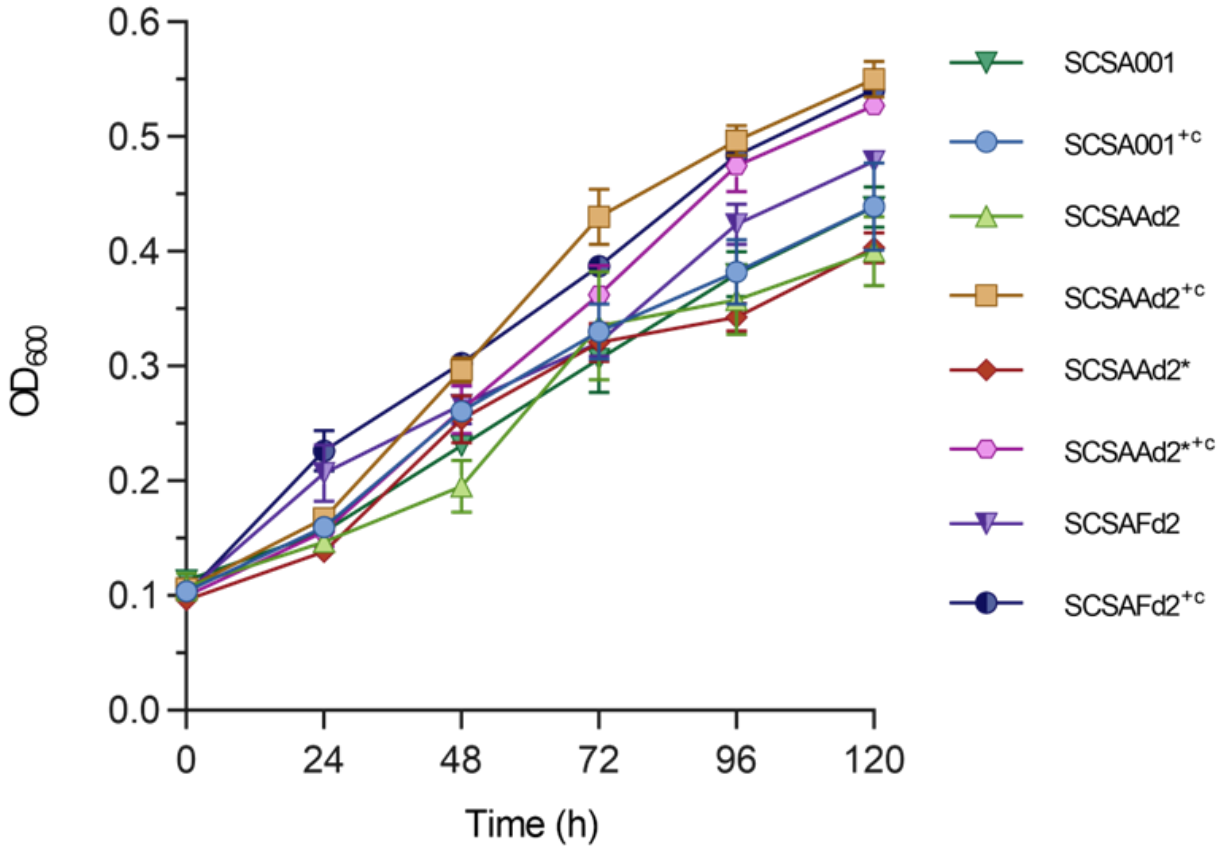


Fig. S22. Growth curves of strain SCSA001, SCSAAd2, SCSAAd2* and SCSAFd2 in Delft MM containing 2% methanol with or without C_3N_4 QDs addition. All strains were precultured in YPD medium and washed twice using PBS (pH = 7.2) before cultivated in Delft MM. All data represent the mean ± s.d. (n = 3 biologically independent samples).

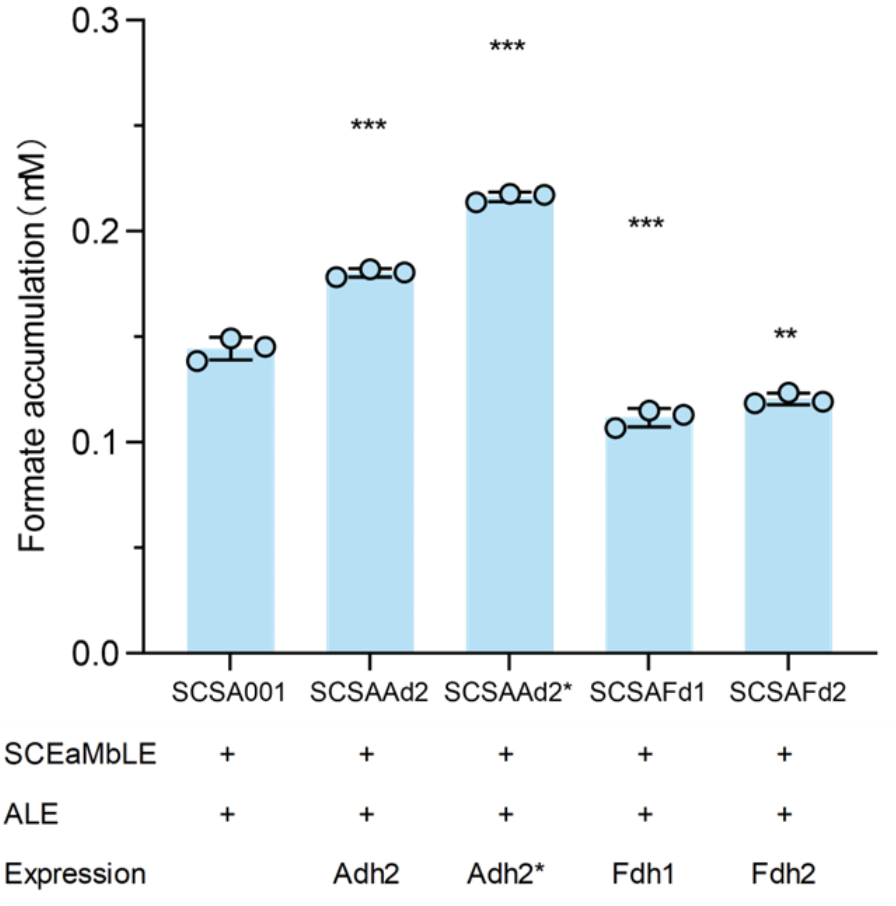


Fig. S23. Formate accumulation in Delft MM with 2% methanol. All strains were precultured in YPD medium and washed twice using PBS (pH = 7.2) before cultivated in Delft MM. All data represent the mean ± s.d. (n = 3 biologically independent samples). Statistical analysis was performed using a two-tailed Student's t-test (**$P < 0.01$, ***$P < 0.001$).

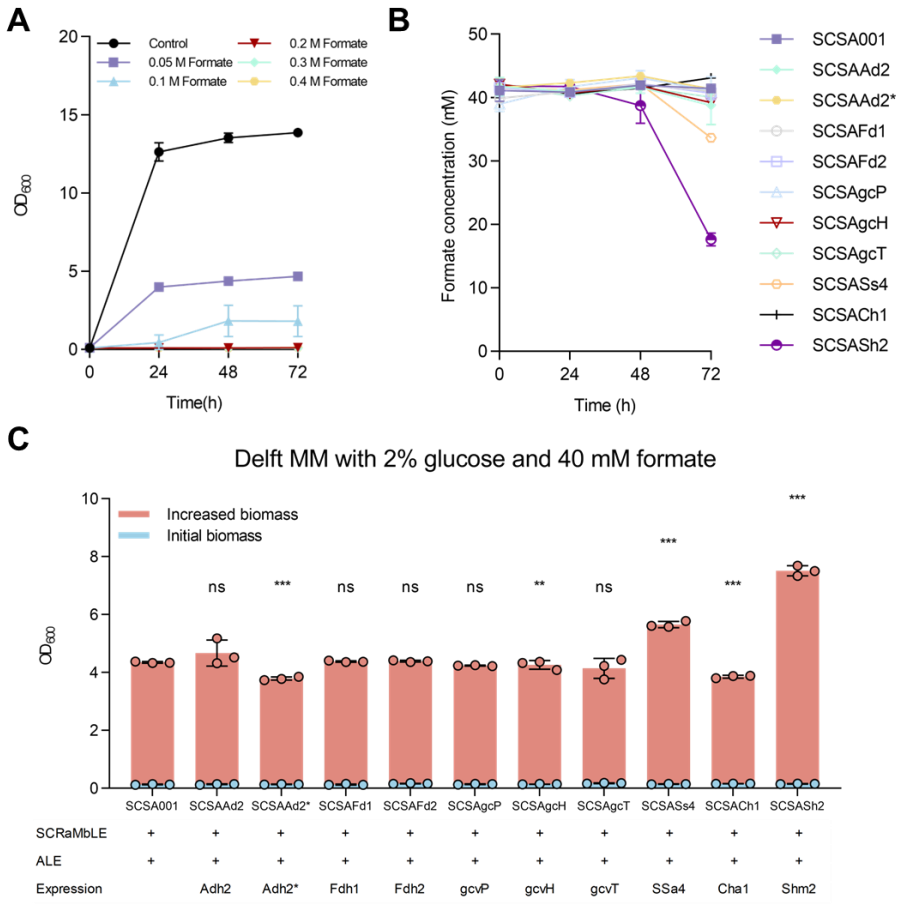


Fig. S24. Evaluation of the formate detoxification capacity of the ASrG pathway. (A) Growth curves of strain SCSA001 on Delft MM containing 20 g/L glucose and different concentrations of formate. (B) Formate consumption curve of strains on Delft MM containing 20 g/L glucose and 40 mM formate. (C) Biomass (OD_{600}) formation on Delft MM with 2% glucose and 40 mM formate. All strains were precultured in YPD medium and washed twice using PBS (pH = 7.2) before cultivated in Delft MM. All data represent the mean ± s.d. (n = 3 biologically independent samples). Statistical analysis was performed using a two-tailed Student's t-test (**P < 0.01, ***P < 0.001). All data are presented as mean ± s.d. Triplicate independent samples were adopted for each panel.

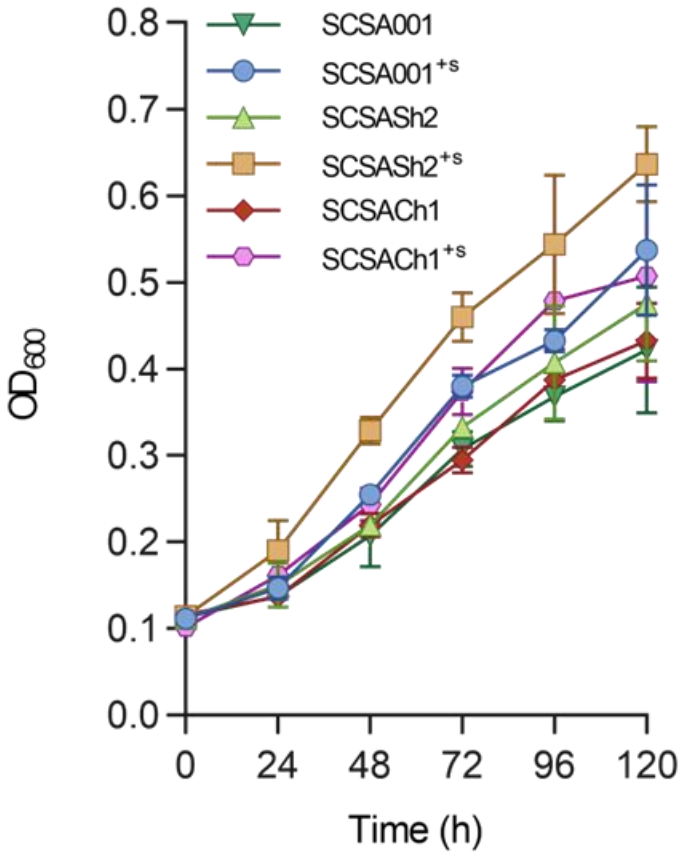


Fig. S25. Growth curves of strain SCSA001, SCSASh2 and SCSACh1 in Delft MM containing 2% methanol with or without serine addition. All strains were precultured in YPD medium and washed twice using PBS (pH = 7.2) before cultivated in Delft MM. All data represent the mean ± s.d. (n = 3 biologically independent samples).

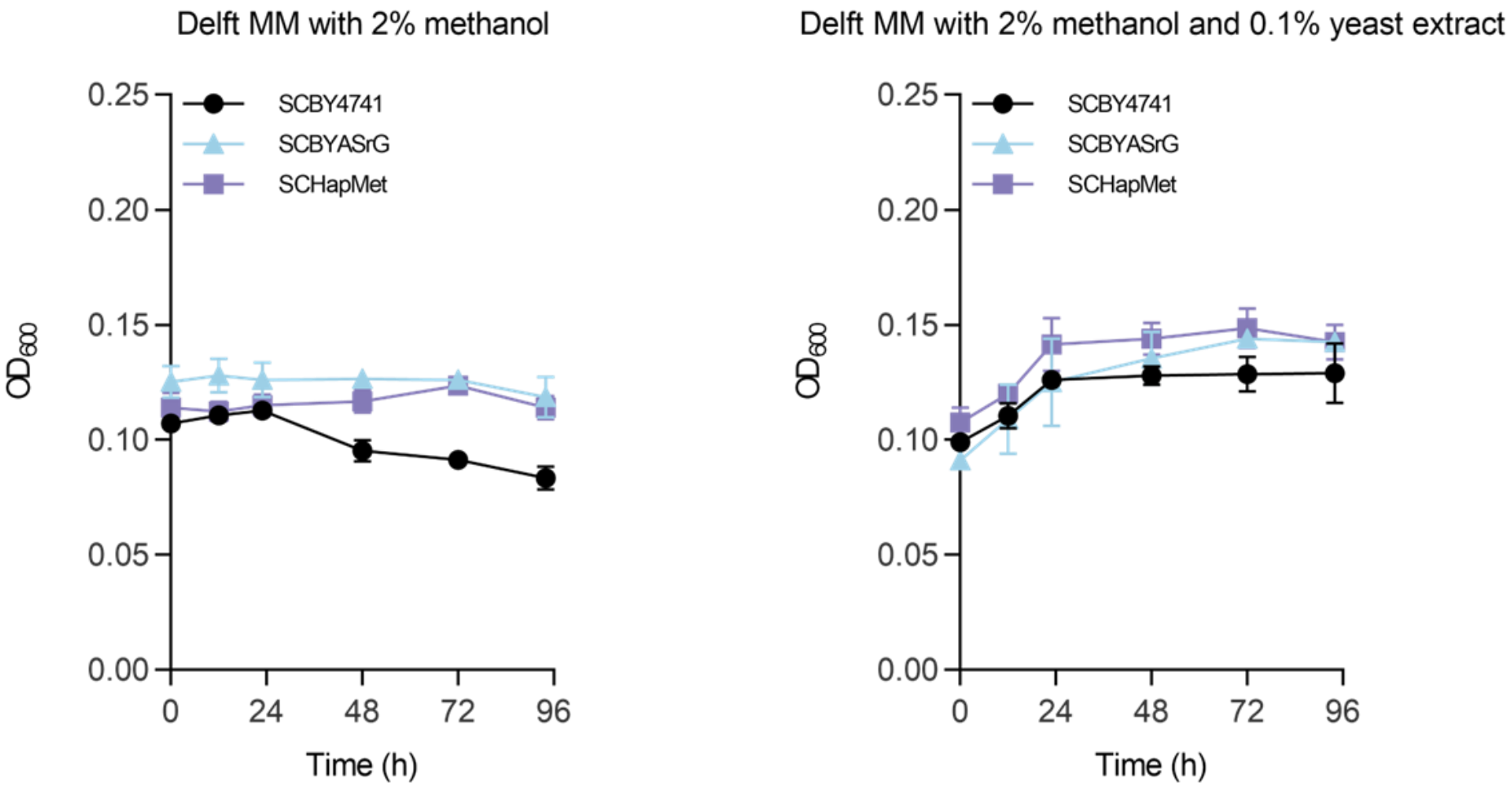


Fig. S26. Growth curves of *S. cerevisiae* SCBY4741, SCBYASrG, and SCHapMet in Delft MM containing 2% methanol. For strain SCBY4741 and SCBYASrG, 20 mg/l uracil, 20 mg/l histidine, 100 mg/l leucine, and 20 mg/l methionine were supplemented to compensate for auxotroph; for strain SCHapMet, 20 mg/l uracil was supplemented to compensate for uracil auxotroph. All data are presented as mean ± s.d. Triplicate independent samples were adopted for each panel.

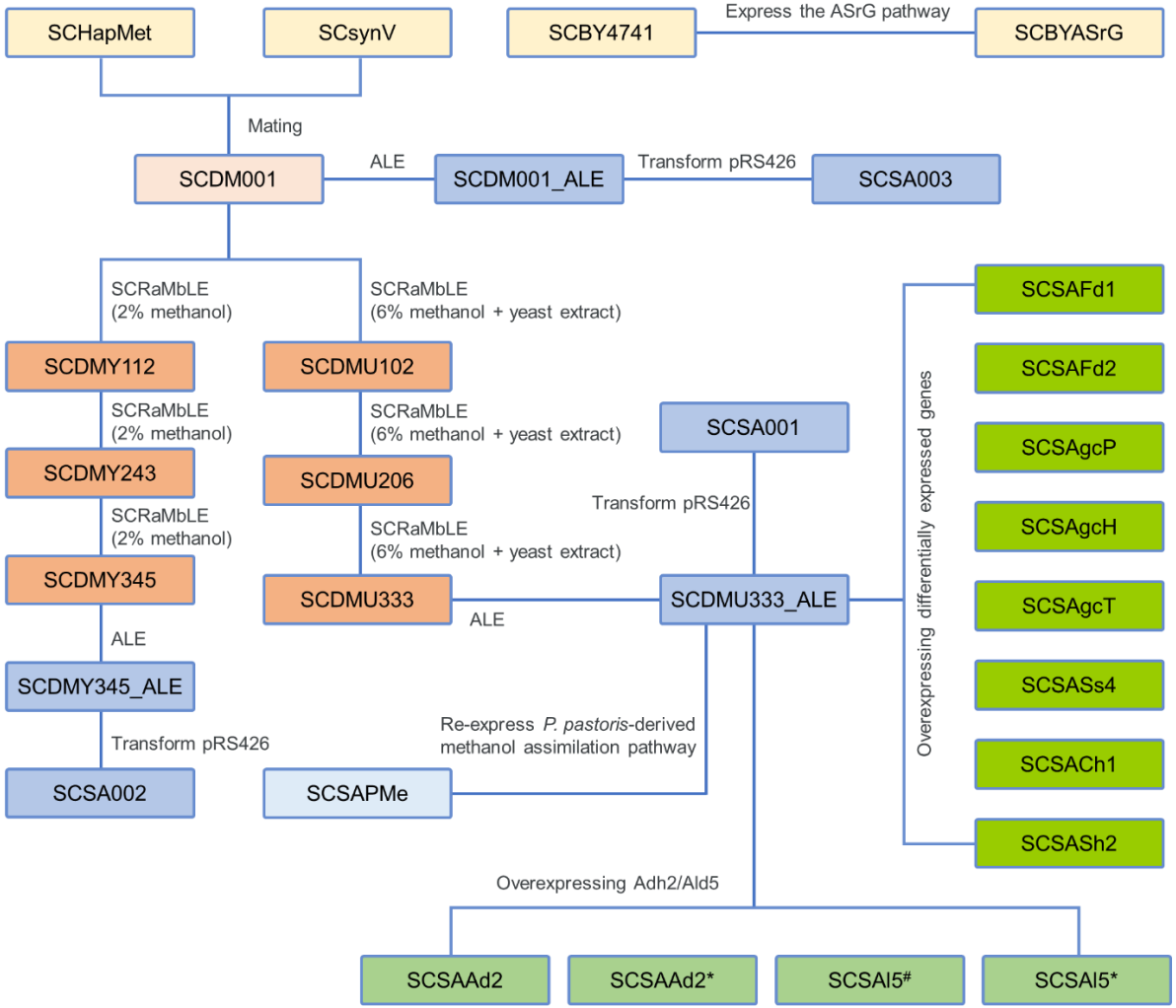


Fig. S27. Flowchart of *S. cerevisiae* construction in this study.

Supplementary Table

Table S1. Thermodynamic calculation of the ASrG pathway.

Steps	Reactions	$\Delta_rG'^m$ (kJ/mol)
1	NAD^+(aq) + Methanol(aq) ⇌ NADH(aq) + Formaldehyde(aq)	42.2 ± 6.5
2	Formaldehyde(aq) + Glutathione(aq) ⇌ S-(Hydroxymethyl)glutathione(aq)	6.4 ± 5.6
3	S-(Hydroxymethyl)glutathione(aq) + NAD^+(aq) ⇌ S-Formylglutathione(aq) + NADH(aq)	-20.6 ± 9.2
4	S-Formylglutathione(aq) + H_2O(l) ⇌ Formate(aq) + Glutathione(aq)	-16.9 ± 8.7
5	ATP(aq) + Formate(aq) + Tetrahydrofolate(aq) ⇌ ADP(aq) + Orthophosphate(aq) + 10-Formyltetrahydrofolate(aq)	-6.7 ± 3.8
6	10-Formyltetrahydrofolate(aq) ⇌ H_2O(l) + 5,10-Methenyltetrahydrofolate(aq)	1.3 ± 1.8
7	NADPH(aq) + 5,10-Methenyltetrahydrofolate(aq) ⇌ NADP+(aq) + 5,10-Methylenetetrahydrofolate(aq)	13.2 ± 2.2
8	Formate(aq) + NAD^+(aq) ⇌ CO_2(aq) + NADH(aq)	-11.7 ± 6.4
9	NADH(aq) + NH_3(aq) + 5,10-Methylenetetrahydrofolate(aq) + CO_2(aq) ⇌ NAD^+(aq) + Glycine(aq) + Tetrahydrofolate(aq)	-8.1 ± 7.1
10	5,10-Methylenetetrahydrofolate(aq) + Glycine(aq) + H_2O(l) ⇌ Tetrahydrofolate(aq) + L-Serine(aq)	6.6 ± 2.4
11	L-Serine(aq) ⇌ NH_3(aq) + Pyruvate(aq)	-23.6 ± 1.8
Total	3 Methanol + 2 H_2O + 2 ATP + 4 NAD^+ ⇌ Pyruvate + 2 ADP + 4 NADH	-34.7
	2 Methanol + CO_2 + H_2O + 2 ATP + 1 NAD^+ ⇌ Pyruvate + 2 ADP + NADH	-7.4
	3 Formate + 2 ATP + 2 NADH ⇌ Pyruvate + H_2O + 2 ADP + 2 NAD^+	-44.6
	2 Formate + CO_2 + 2 ATP + 3 NADH ⇌ Pyruvate + H_2O + 2 ADP + 3 NAD^+	-32.9

The reaction Gibbs energy was calculated through the eQuilibrator biochemical thermodynamics calculator. $\Delta_rG'^m$ (KJ/mol)) is the free Gibbs energy when the reactant concentration was set to be 1 mM under the condition of pH=6.0, pMg=3.0, and 0.1 M ionic strength (*78*).

Table S2. Strains used in this study.

Strain Name	Description	Genotype[$]	Source
SCBY4741	/	*MATa his3Δ1 leu2 met15Δ ura3-52 wtV*	Invitrogen
SCHapMet	haploid, containing *P. pastoris* methanol assimilation pathway	*MATα* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA}) *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 wtV*	Previously constructed (*22*)
SCsynV	haploid, synV	*MATa*, *synV*	(*10*)
SCDM001	SCHapMet mating SCsynV	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA}) *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV*	This study
SCDMU102	SCDM001, one round SCRaMbLE (2% methanol)	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**	This study
SCDMU206	SCDM001, two rounds SCRaMbLE (2% methanol)	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**	This study
SCDMU333	SCDM001, three rounds SCRaMbLE (2% methanol)	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**	This study
SCDMY112	SCDM001, one round SCRaMbLE (6% methanol + 0.1 g/l yeast extract)	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**	This study
SCDMY243	SCDM001, two rounds SCRaMbLE (6% methanol + 0.1 g/l yeast extract)	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**	This study
SCDMY345	SCDM001, three rounds SCRaMbLE (6% methanol + 0.1 g/l yeast extract)	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**	This study
SCDM001_ALE	SCDM001, ALE	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **ALE**	This study
SCDMU333_ALE	SCDMU333, ALE	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**, **ALE**	This study
SCDMY345_ALE	SCDMY345, ALE	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx ura3-52 synV/wtV* **SCRaMbLE**, **ALE**	This study
SCSA001	SCDMU333_ALE, pRS426	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**	This study
SCSA002	SCDMY345_ALE, pRS426	*MATa/α* AOX(P_{PGK})-DAS2(P_{TDH})-CAT(P_{PDC})-DAK(P_{FBA})[&] *pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**	This study
SCSA003	SCDM001_ALE, pRS426	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV*	This study

		ALE	
SCSAPMe	SCSA001, overexpressing *P. pastoris*-derived methanol assimilation pathway	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{PGK1}-*Pp*Aox1(ePTS)-T_{CYC1}-P_{TDH3}-*Pp*Das2(ePTS)-T_{PGK1}-P_{PDC1}-*Pp*Cta(ePTS)-T_{TDH2}-P_{FBA1}-*Pp*Dak(ePTS)-T_{ENO2})	This study
SCSAAd2	SCSA001, overexpressing Adh2	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Adh2-T_{ENO2})	This study
SCSAAd2*	SCSA001, overexpressing Adh2*	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Adh2*-T_{ENO2})	This study
SCSAAl5$^{\#}$	SCSA001, overexpressing Ald5$^{\#}$	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Ald5$^{\#}$-T_{ENO2})	This study
SCSAAl5*	SCSA001, overexpressing Ald5*	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Ald5*-T_{ENO2})	This study
SCSAFd1	SCSA001, overexpressing Fdh1	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Fdh1-T_{ENO2})	This study
SCSAFd2	SCSA001, overexpressing Fdh2	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Fdh2-T_{ENO2})	This study
SCSAgcP	SCSA001, overexpressing gcvP	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-gcvP-T_{ENO2})	This study
SCSAgcH	SCSA001, overexpressing gcvH	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-gcvH-T_{ENO2})	This study
SCSAgcT	SCSA001, overexpressing gcvT	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-gcvT-T_{ENO2})	This study
SCSASs4	SCSA001, overexpressing Ssa4	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Ssa4-T_{ENO2})	This study
SCSACh1	SCSA001, overexpressing Cha1	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Cha1-T_{ENO2})	This study
SCSASh2	SCSA001, overexpressing Shm2	*MATa/α pdc1(-6,-2)::loxP pdc5(-6,-2)::loxP pdc6(-6,-2)::loxP mutx synV/wtV* **SCRaMbLE**, **ALE**, pRS426 (P_{TEF1}-Shm2-T_{ENO2})	This study
SCBYASrG	SCBY4741, overexpressing the ASrG pathway	*MATa* Adh2*(P_{TEF1})-Fdh2(P_{PGK1})-GcvP(P_{TDH})-GcvH(P_{PDC})-GcvT(P_{FBA}) *his3Δ1 leu2 met15Δ ura3-52 wtV*	This study
PPGS115	*Komagataella phaffii* GS115	*Mut^{+}, his4^{-}, Aox1, Aox2*	This study
PPAdh2	PPGS115, overexpressing Adh2	*Mut^{+}, his4^{-}, Aox1, Aox2*, AOX1::P_{GAP}-Adh2-T_{Aox1}	This study

PPAdh2*	PPGS115, overexpressing Adh2*	*Mut*$^{+}$*, his4*$^{-}$*, Aox1, Aox2*, AOX1::P_{GAP}-Adh2*-T_{Aox1}	This study

[$] The genotype changes caused by SCRaMbLE and ALE cannot be fully described in this table. Detailed genotypic changes were shown in **Supplementary Data**.
[&] It is uncertain whether the *P. pastoris* methanol assimilation pathway is present in the genomes of these strains as whole genome sequencing was not performed on these strains.

Table S3. Plasmids used in this study.

Plasmid name	Genotype	Source
pLM050-pCLB2-CreEBD	pRS413, pCLB2-Cre-EBD-tCYC1, CEN/ARS with *His3* marker	(*17*)
pLM050-pCLB2-CreEBD-Hph	pRS413, pCLB2-Cre-EBD-tCYC1, CEN/ARS with *His3* and hygromycin marker	This study
pRS426	*Ura3* marker	Invitrogen
pRS426-Adh2	pRS426, P_{TEF1}-Adh2-T_{ENO2}	This study
pRS426-Adh2*	pRS426, P_{TEF1}-Adh2*-T_{ENO2}	This study
pRS426-Ald5$^{\#}$	pRS426, P_{TEF1}-Ald5$^{\#}$-T_{ENO2}	This study
pRS426-Ald5*	pRS426, P_{TEF1}-Ald5*-T_{ENO2}	This study
pRS426-Fdh1	pRS426, P_{TEF1}-Fdh1-T_{ENO2}	This study
pRS426-Fdh2	pRS426, P_{TEF1}-Fdh2-T_{ENO2}	This study
pRS426-gcvP	pRS426, P_{TEF1}-gcvP-T_{ENO2}	This study
pRS426-gcvH	pRS426, P_{TEF1}-gcvH-T_{ENO2}	This study
pRS426-gcvT	pRS426, P_{TEF1}-gcvT-T_{ENO2}	This study
pRS426-Ssa4	pRS426, P_{TEF1}-Ssa4-T_{ENO2}	This study
pRS426-Cha1	pRS426, P_{TEF1}-Cha1-T_{ENO2}	This study
pRS426-Shm2	pRS426, P_{TEF1}-Shm2-T_{ENO2}	This study
pRS426-PpMet	pRS426, P_{PGK1}-*Pp*Aox1(ePTS)-T_{CYC1}-P_{TDH3}-*Pp*Das2(ePTS)-T_{PGK1}-P_{PDC1}-*Pp*Cta(ePTS)-T_{TDH2}-P_{FBA1}-*Pp*Dak(ePTS)-T_{ENO2}	This study
pGAPZ(alpha) B	Zeocin marker, alpha-Factor	Invitrogen
pGAPZ(alpha) B-Adh2	pGAPZ(alpha) B, P_{GAP}-Adh2-T_{Aox1}	This study
pGAPZ(alpha) B-Adh2*	pGAPZ(alpha) B, P_{GAP}-Adh2*-T_{Aox1}	This study

Table S4. Primers used in this study.

Primer name	Sequence (5'-3')
TEF-HPH F	AATGTTTCTACTCCTTTTTTACTCTTC
HPH-CYC1tt R	GCAAATTAAAGCCTTCGAGC
EcoR1-pTEF F	GATAAGCTTGATATCGAATTCAATGTTTCTACTCCTTTTTTACTCTTCCA
CYC1tt -EcoR1 R	ATTGGCGGGCTGCAGGAATTCGCAAATTAAAGCCTTCGAGCG
Cre-hph cof F	CTGATCGAAAAGTTCGACAGCG
Cre-hph cof R	TCTGACAAACGAGCGAATGAAG
YEL056W1 F	CTCACACCACAGTAGCAATTTGTTGGCT
YEL056W1 R	GCCACCAACAATTGGCAATGAGTGACTG
YEL038W1 F	TCCAGCTCACGACAGCTTAGACTTAAAC
YEL038W1 R	AACTGGAGCGTTGCCTGGTCTACTG
YEL022W3 F	GACCATTGGCTTATTGTTGTGTCACCCA
YEL022W3 R	ATTACCATGATGCTCTTCTGGCATGACG
YER026C1 F	TGATTTGCTGATCATACCGCAACCGTGA
YER026C1 R	CGGCAAGCCACACTATGTTCAGAGAGCT
YER043C1 F	GCAGCCGGCGATTCTAGCACCCTTTAAT
YER043C1 R	TGGTTGCCGTGAAAGTTTGGTTGATGGC
YER056C2 F	AAAAGCCAAGCCGGCGACCAAACTAAAG
YER056C2 R	CGCTTATGAGAAGTGGAGCTGGGTTCCA
YER070W2 F	GCGTCCAGGCGCTTTCGCTTTGTATTTA
YER070W2 R	CTTCTCATATCTAGTGTACAAGGCCTCG

YER086W1 F	TAGCCAAGGCGTTGGCTTAAGCAGTAGA
YER086W1 R	CAAAACAACTTGGCTACCCAAACGGCTA
YER100W1 F	CCCAGGTTGGAGCGTTAGCACTATCTTA
YER100W1 R	AGCTTGTTCGGCTCTGATACGATCTTCT
YER113C2 F	ACCAACGCTGTTAGCCAATGACATCCAG
YER113C2 R	CCACTGCCCAGGTGCTAGCAAAAATTAT
YER144C2 F	AGTGCTTGGGGTGTTCAACTTGCTCAAG
YER144C2 R	CAGCGTTATCAAGCCATTATCAGGTACC
YER149C1 F	ACTGCTTCTAGCACGCTTGCTGTTAGGA
YER149C1 R	TTTGTTGAGTCAAAACCCAAGCCACCCA
YER172C6 F	GCTACCTTCTGGCAAACTAACCTTGGTA
YER172C6 R	GTTGAGAAGCGAGTTGGGCTATAAAGAC
YER188W1 F	TCGTAGAAGCGACGCTTTGGGTGTTACC
YER188W1 R	GTTCCAACACAACATCTGACCAGGTCTG
MATa F	ACTCCACTTCAAGTAAGAGTTTG
MATa/α R	AGTCACATCAAGATCGTTTATGG
MATα F	GCACGGAATATGGGACTACTTCG
AOX1 cof F	ATGGCTATCCCCGAAGAGTTTG
AOX1 cof R	TTAGAATCTAGCAAGACCGGTCTTC
DAS1 cof F	ATGGCTAGAATTCCAAAAGCAGTAT
DAS1 cof R	TTACAACTTGTCATGCTTTGGTTTT
CTA cof F	ATGTCTCAACCACCTAAATGGACA

CTA cof R	CTACAATCTTGCTGCAGAGTCACC
DAK cof F	ATGTCTAGTAAACATTGGGATTACAAGAA
DAK cof R	CTACAACTTGGTTTCAGATTTGAAGTATG
NS1	GTAGTCATATGCTTGTCTC
NS8	TCCG-CAGGTTCACCTACGGA
Sac1-TEF1p F	GCCGCCACCGCGGTGGAGCTGATCCCCCACACACCATAGCT
TEF1p-ENO2tt R	CTTAGTTAAAAGCACTGAGCTCTTGTAATTAAAACTTAGATTAGATTGCTATGC
TEF1p-ENO2tt F	ACAAGAGCTCAGTGCTTTTAACTAAGAATTATTAGTCTTTTC
ENO2tt-Sac1 R	AGGGAACAAAAGCTGGAGCTAGGTATCATCTCCATCTCCCATATG
Sac1-PAC F	GCCGCCACCGCGGTGGAGCTCTATTTTAGATTCCTGACTTCAACTCAAGA
PAC-TDP R	GCAAATTAAAGCCTTCGAGCG
PAC-TDP F	GCTCGAAGGCTTTAATTTGCATAAAAAACACGCTTTTTCAGTTCG
TDP-PCT R	CTTAGGTTTAACGAACGCAGAATTTTC
TDP-PCT F	CTGCGTTCGTTAAACCTAAGTAACCTATTCAAAGTAATATCTCATACATG
PCT-FDE R	GCGAAAAGCCAATTAGTGTGATAC
PCT-FDE F	CACACTAATTGGCTTTTCGCATAACAATACTGACAGTACTAAATAATTGCC
FDE-Sac1 R	AGGGAACAAAAGCTGGAGCTCAGGTATCATCTCCATCTCCCATATG
Aox1-ePTS F	CTTACGAGAAGACCGGTCTTGCTAGATTCTTGGGAAGAGGTAGAAGATCTAAGTTGTAA
Aox1-ePTS R	TAAGCGTGACATAACTAATTACATGATTACAACTTAGATCTTCTACCTCTTCCCAA
Das2-ePTS F	AGGGAAAACCAAAGCATGACAAGTTGTTGGGAAGAGGTAGAAGATCTAAGTTGTAA
Das2-ePTS R	TCTATCGATTTCAATTCAATTCAATTTTTACAACTTAGATCTTCTACCTCTTCCCAA
Cta-ePTS F	CCAAGAGGTGACTCTGCAGCAAGATTGTTGGGAAGAGGTAGAAGATCTAAGTTGTAG

Cta-ePTS R	AAATCATTAAAGTAACTTAAGGAGTTAAATCTACAACTTAGATCTTCTACCTCTTCCCAA
Dak-ePTS F	GCATACTTCAAATCTGAAACCAAGTTGTTGGGAAGAGGTAGAAGATCTAAGTTGTAG
Dak-ePTS R	AAAGACTAATAATTCTTAGTTAAAAGCACTCTACAACTTAGATCTTCTACCTCTTCCCAA
Sac1-ADH2	AAGTTTTAATTACAAGAGCTCATGTCTATTCCAGAAACTCAAAAAGC
ADH2-Sac1 R	TTAGTTAAAAGCACTGAGCTCTTATTTAGAAGTGTCAACAACGTATCTACC
Sac1-ALD5 F	AAGTTTTAATTACAAGAGCTCATGCTTTCTCGCACAAGAGCTG
ALD5$^{\#}$-Sac1 R	TTAGTTAAAAGCACTGAGCTCTCAACGAATTGGCTTGTCAATG
ALD5*-Sac1 R	TTAGTTAAAAGCACTGAGCTCCTACACCAGCGGGTATGCCT
Sac1-CHA1 F	AAGTTTTAATTACAAGAGCTCATGTCGATAGTCTACAATAAAACACCATT
CHA1-Sac1 R	TTAGTTAAAAGCACTGAGCTCTCAAGCACTTTTTAAATTCACAATATTT
Sac1-FDH1 F	AAGTTTTAATTACAAGAGCTCATGTCGAAGGGAAAGGTTTTGC
FDH1-Sac1 R	TTAGTTAAAAGCACTGAGCTCTTATTTCTTCTGTCCATAAGCTCTGG
Sac1-FDH2 F	AAGTTTTAATTACAAGAGCTCATGTCGAAGGGAAAGGTTTTGC
FDH2-Sac1 R	TTAGTTAAAAGCACTGAGCTCTTATTTCTTCTGTCCATAAGCTCTGG
Sac1-gcvH F	AAGTTTTAATTACAAGAGCTCATGTTACGCACTACTAGACTATGGACC
gcvH-Sac1 R	TTAGTTAAAAGCACTGAGCTCTCAGTCATCATGAACCAGTGTCTTT
Sac1-gcvP F	AAGTTTTAATTACAAGAGCTCATGCTTAGGACAAGAGTGACTGCTC
gcvP-Sac1 R	TTAGTTAAAAGCACTGAGCTCTCATTCAGTTTCGTTCGCAATT
Sac1-gcvT F	AAGTTTTAATTACAAGAGCTCATGTCTATAATCAAAAAAATTGTGTTTAAGA
gcvT-Sac1 R	TTAGTTAAAAGCACTGAGCTCTTACTGCTTGTAGTAATGTGTGGGC
Sac1-HSP F	AAGTTTTAATTACAAGAGCTCATGTCAAAAGCTGTTGGTATTGATTT
HSP-Sac1 R	TTAGTTAAAAGCACTGAGCTCCTAATCAACCTCTTCAACCGTTGG

Sac1-SHMT F	AAGTTTTAATTACAAGAGCTCATGCCTTACACTCTATCCGACGC
SHMT-Sac1 R	TTAGTTAAAAGCACTGAGCTCTTACACAGCCAATGGGTATTCG
prs426 cof F	CATTGATATTTAAGTTAATAAACGGTCTTC
prs426 cof R	TATTCGTTAATATAAAGTGTTCTAAACTATGATG
EcoR1-Adh2 F	AGGCTGAAGCTGCAGGAATTCATGTCTATTCCAGAAACTCAAAAAGC
Adh2-EcoR1 R	CCGGCTGGGCCACGTGAATTCTTATTTAGAAGTGTCAACAACGTATCTACC
Delta1-KanMX F	TGTTGGAATAAAAATCAACTATCGGCTGGCAACTAATAGGGACACTACCA
Delta1-KanMX F	ATAGGCCACTAGTGGATCTGATATCA
Adh2* F	CAGATCCACTAGTGGCCTATTTGTAATTAAAACTTAGATTAGATTGCTATGC
Adh2* R	GGTATACTGGAGGCTTCATGAGTTATG
Fdh2 F	CATGAAGCCTCCAGTATACCTATTTTAGATTCCTGACTTCAACTCAAGA
Fdh2 R	GCAAATTAAAGCCTTCGAGCG
GcvP F	GCTCGAAGGCTTTAATTTGCATAAAAAACACGCTTTTTCAGTTCG
GcvP R	CTTAGGTTTAACGAACGCAGAATTTTC
GcvH F	CTGCGTTCGTTAAACCTAAGTAACCTATTCAAAGTAATATCTCATACATG
GcvH R	GCGAAAAGCCAATTAGTGTGATAC
GcvT F	CACACTAATTGGCTTTTCGCATAACAATACTGACAGTACTAAATAATTGCC
GcvT R	AAAAAAAAGTTCCGAGTAATTAATGTTGAGATATGTTGGAATAAAAATCAAC

Table S5. Sequences of mutant genes used in this study.

Gene	Sequence
Adh2*	ATGTCTATTCCAGAAACTCAAAAAGCCATTATCTTCTACGAATCCAACGGCAAGTTGGAGCATAAGGATATCCCAGTTCCAAAGCCAAAG CCCAACGAATTGTTAATCAACGTCAAATACTCTGGTGTCTGCCACACCGATTTGCACGCTTGGCATGGTGACTGGCCATTGCCAACTAAG TTACCATTAGTTGGTGGGCACGAAGGTGCTGGGGTCGTTGTTGCTATCGGGGACAACGTTAGGGGTTGGAAAGTGGGTGATCTTGCCGG TATTAAATGGTTGAACAGTTCATGTATGGCCTGCGAATACTGTGAATTGGGTAACGAATCCAACTGTCCTCACGCTGACTTGTCTGGTTAC ACCCATGACGGTTCTTTCCAACAATACGCTACTGCTGACGCGGTGCAAGCCGCTCGTATTCCCGAAGGGACCGACTTGGCCCAAGTCGC CCCCATCTTGTGTGCTGGTATCACCGTATACAAGGCTTTGAAGTCTGCCAACTTGAGAGCAGGCCACTGGGTGGCCATTTCTGGTGCTGC TGGTGGTCTAGGTTCTTTGGCTGTTCAATATGCTAAGGCGATGGGTTACAGAGTCTTAGGTATTGATGGTGGTCCAGGAAAGGAAGAATT GTTTACCTCGCTCGGTGGTGAAGTATTCATCGACTTCACCAAAGAGAAGGACATTGTTAGCGCAGTCGTTAAGGCTACCAACGGCGGTG CCCACGGTATCATCAATGTTTCCGTTTCCGAAGCCGCTATCGAAGCTTCTACCAGATACTGTAGGGCGAACGGTACTGTTGTCTTGGTTGG TTTGCCAGCCGGTGCAAAGTGCTCCTCTGATGTCTTCAACCACGTTGTCAAGTCTATCTCCATTGTCGGCTCTTACGTGGGGAACAGAGC TGATACCAGAGAAGCCTTAGATTTCTTTGCCAGAGGTCTAGTCAAGTCTCCAATAAAGGTAGTTGGCTTATCCAGTTTACCAGAAATTTAC GAAAAGATGGAGAAGGGCCAAATTGCTGGTAGATACGTTGTTGACACTTCTAAATAA
Ald5*	ATGCTTTCTCGCACAAGAGCTGCAGCTCCGAATTCCAGAATATTCACTAGAAGCTTGTTACGTCTTTATTCTCAAGCACCATTACGCGTTC CAATTACTCTTCCAAATGGTTTCACCTACGAACAGCCAACAGGGTTATTCATCAATGGTGAATTTGTTGCCTCGAAGCAAAAGAAAACGT TTGACGTGATCAATCCATCTAACGAAGAAAAGATAACAACTGTATACAAGGCTATGGAAGATGATGTTGATGAAGCCGTTGCAGCGGCTA AAAAAGCTTTTGAAACGAAGTGGTCTATTGTAGAGCCGGAGGTTCGCGCTAAAGCTTTATTCAATCTCGCTGACTTGGTTGAGAAACAC CAAGAAACACTGGCTGCCATTGAGTCAATGGATAATGGTAAGTCATTGTTTTGTGCGCGCGGTGACGTCGCTTTAGTATCTAAATACTTGC GTTCTTGCGGTGGTTGGGCAGATAAAATCTACGGTAACGTTATTGACACAGGTAAAAACCATTTTACCTACTCAATTAAGGAACCATTAG GCGTTTGCGGCCAAATAATCCCTTGGAACTTCCCTTTATTGATGTGGTCATGGAAAATTGGGCCTGCTCTGGCTACAGGTAACACCGTCGT ATTGAAACCCGCTGAAACCACCCCATTGAGCGCTTTGTTTGCCAGTTGTGTCAGGAAGCAGGCATACCCGCTGGTGTAG
Ald5#	ATGCTTTCTCGCACAAGAGCTGCAGCTCCGAATTCCAGAATATTCACTAGAAGCTTGTTACGTCTTTATTCTCAAGCACCATTACGCGTTC CAATTACTCTTCCAAATGGTTTCACCTACGAACAGCCAACAGGGTTATTCATCAATGGTGAATTTGTTGCCTCGAAGCAAAAGAAAACGT TTGACGTGATCAACCCATCTAACGAAGAAAAGATAACAACTGTATACAAGGCTATGGAAGACGATGTTGATGAAGCCGTTGCAGCGGCT AAAAAAGCTTTTGAAACGAAGTGGTCTATTGTAGAGCCGGAGGTTCGCGCTAAAGCTTTATTCAATCTCGCTGACTTGGTTGAGAAACA CCAAGAAACACTGGCTGCCATTGAGTCAATGGATAATGGTAAGTCATTGTTTTGTGCGCGCGGTGACGTCGCTTTAGTATCTAAATACTTG CGTTCTTGCGGTGGTTGGGCAGATAAAATCTACGGTAACGTTATTGACACGGGTAAAAACCATTTTACCTACTCAATTAAGGAACCATTAG GCGTTTGCGGCCAAATAATCCCTTGGAACTTCCCTTTATTAATGTGGTCATGGAAAATTGGGCCTGCTCTGGCTACAGGTAACACCGTCGT ATTGAAACCCGCTGAAACAACACCTTTATCTGCCCTTTTCGCTTCCCAGTTGTGTCAGGAAGCAGGCATACCCGCTGGTGTAGTCAATAT CCTTCCGGGTTCCGGTAGAGTTGTTGGAGAAAGATTGAGTGCACACCCAGACGTGAAGAAGATTGCTTTTACAGGCTCTACTGCCACCG GCCGCCATATTATGAAGGTCGCTGCCGATACTGTCAAGAAAGTCACTTTGGAGCTGGGAGGTAAATCACCAAATATTGTGTTTGCTGACG CTGATTTAGATAAAGCCGTTAAGAACATTGCCTTCGGTATTTTTTACAACTCCGGTGAAGTTTGCTGTGCTGGTTCCAGAATATACATTCA

AGATACAGTATACGAGGAGGTGTTGCAAAAACTAAAGGATTACACCGAGTCACTAAAGGTCGGTGACCCATTTGATGAGGAAGTTTTCC
AAGGTGCTCAAACATCTGACAAACAGCTGCATAAAATTTTAGACTATGTCGATGTAGCAAAATCAGAGGGGGCTCGTCTTGTGACTGGA
GGTGCCAGACATGGCAGTAAAGGTTATTTTGTCAAGCCAACAGTGTTTGCTGATGTCAAAGAAGATATGAGAATTGTTAAGGAGGAAGT
GTTTGGTCCCATTGTAACTGTATCCAAGTTTTCTACTGTTGATGAAGTGATTGCTATGGCAAATGATTCTCAATATGGGTTAGCCGCAGGTA
TTCACACTAACGATATTAACAAGGCTGTTGATGTGTCCAAAAGAGTGAAAGCTGGTACTGTTTGGATAAATACCTATAACAACTTCCACC
AAAATGTTCCTTTCGGTGGCTTCGGCCAGTCAGGTATTGGCCGTGAAATGGGTGAGGCTGCTTTAAGTAACTACACTCAAACAAAATCTG
TCAGAATTGCCATTGACAAGCCAATTCGTTGA

Supplementary Data

Data S1. (separate file)
Processed comparative genomics data. Specific SNP, INDEL, CNV and SV information are listed for strains SCSA001 and SCSA003 relative to the control SCDM001.

Data S2. (separate file)
Processed transcriptome data. Gene id, fold change, $_2$foldchange, *P* value, gene names and functional annotations are listed. The control group (Y_2) was cultured in Delft MM supplemented with 0.1% yeast extract. The test group (MY_2) was cultured in Delft MM supplemented with 0.1% yeast extract and 2% methanol.