Electronic Supplementary Material (ESI) for RSC Advances.

Improving the catalytic performance of Pichia pastoris Whole-cell biocatalysts by fermentation strategies

Supplementary Table 1. Plasmids used or constructed in this study

Plasmid name	Genotype/ Description	Reference
pPICZA	P_{AOX1}-MCS-Myc-6xHis-T_{AOX1}-P_{TEF}1-P_{EM7}-BleoR-T_{CYC1}-ori	
pHKA	P_{AOX1}-α-factor-Myc-Flag-T_{AOX1}-PpHis4-KanR-ori	
pPICZA-1	pPICZA, P_{AOX1}-SIIE -Myc-6xHis-T_{AOX1}	This study
pPICZA-2	pPICZA, 2x(P_{AOX1}-SIIE -Myc-6xHis-T_{AOX1})	This study
pHKA-1	pHKA, P_{AOX1}-α-factor-GCW61-UTG76G1-Myc-Flag-T_{AOX1}	This study
pHKA-2	pHKA, 2x(P_{AOX1}-α-factor-GCW61-UTG76G1-Myc-Flag-T_{AOX1})	This study
pHKA-3	pHKA, 3x(P_{AOX1}-α-factor-GCW61-UTG76G1-Myc-Flag-T_{AOX1})	This study
pHKA-4	pHKA, 4x(P_{AOX1}-α-factor-GCW61-UTG76G1-Myc-Flag-T_{AOX1})	This study
pHKA-5	pHKA, 5x(P_{AOX1}-α-factor-GCW61-UTG76G1-Myc-Flag-T_{AOX1})	This study

Supplementary Table 2. Primers used in this study

Primer name	Sequence (5'-3')
ECORI-mbSUS-1	GCGAATTCATGGCTACTGACAGATT
NotI-mbSUS-2	ATGCGGCCGCCTCAACAGCCAATGGAAC
ECORI-UGT76G1-1	GCATGGAGAATAAGACCGAGACT
UGT76G1-2	GCTTACAAGGAAGAGATATATGAA
NotI+GCW61-1	GAGCGGCCGCTTAAATCAATAGAGCAACAC
GCW61-2	GCACAACCTATCAAACGAGAGTA
UGT76G1+GCW61	TTGTTTCATATATCTCTTCCTTGACGCGTAACAACCTATCAAACGAGAGTAATGGTACTAAT
GCW61+UGT76G1	TCTCGTTTGATAGGTTGTTACGCGTCAAGGAAGAGATATATGAAACAAGGGACTCCAAAGATT

Supplementary Table 3. The optimization experiments of culture temperature, pH value and methanol concentration.

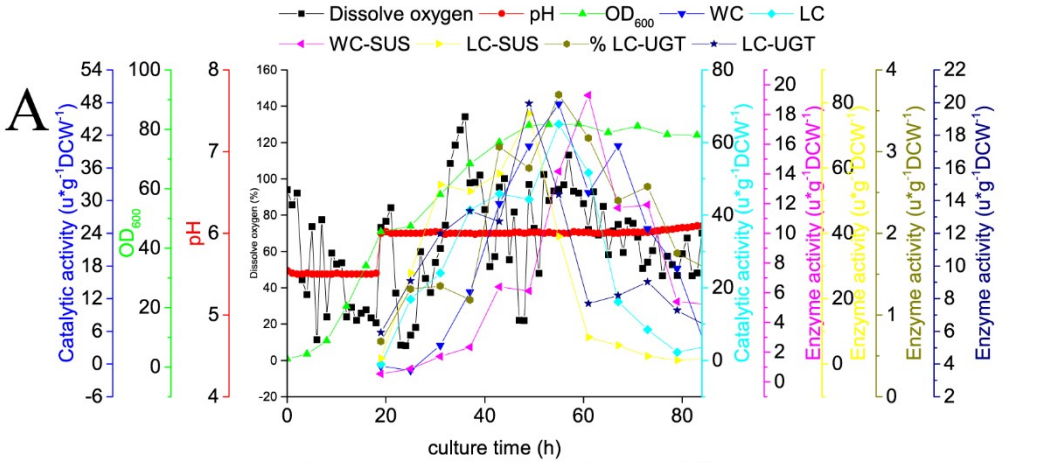
	Temperature (°C)	pH value	Methanol concentration ($mL \cdot L^{-1}$)
Optimization of culture temperature			
Experiment 1	20	6.0	10
Experiment 2	25	6.0	10
Experiment 3	30	6.0	10
Optimization of pH value			
Experiment 4	30	4	10
Experiment 5	30	5	10
Experiment 6	30	6	10
Experiment 7	30	7	10
Experiment 8	30	8	10
Experiment 9	30	9	10
Optimization of methanol concentration			
Experiment 10	30	6	5
Experiment 11	30	6	10
Experiment 12	30	6	20
Experiment 13	30	6	30

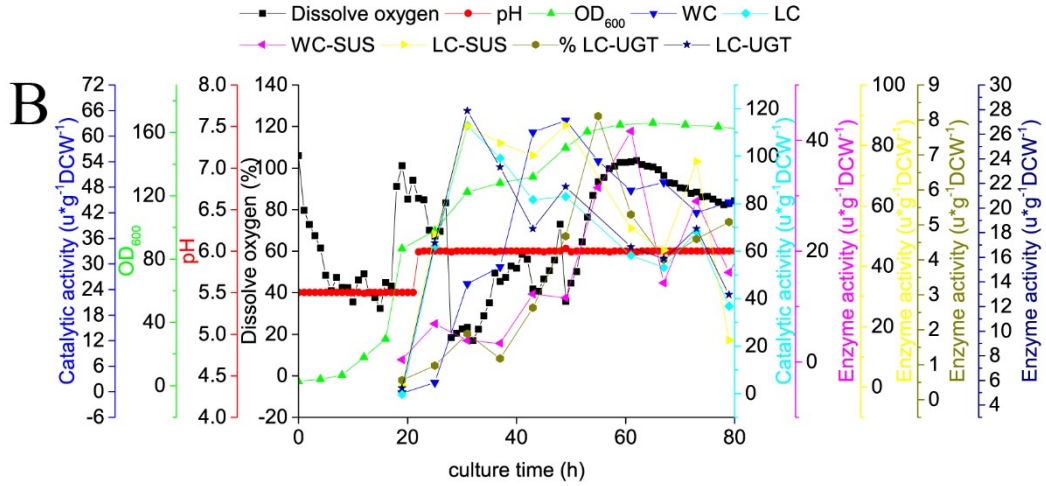
Supplementary Table 4. The Strategies in 15L bioreactor

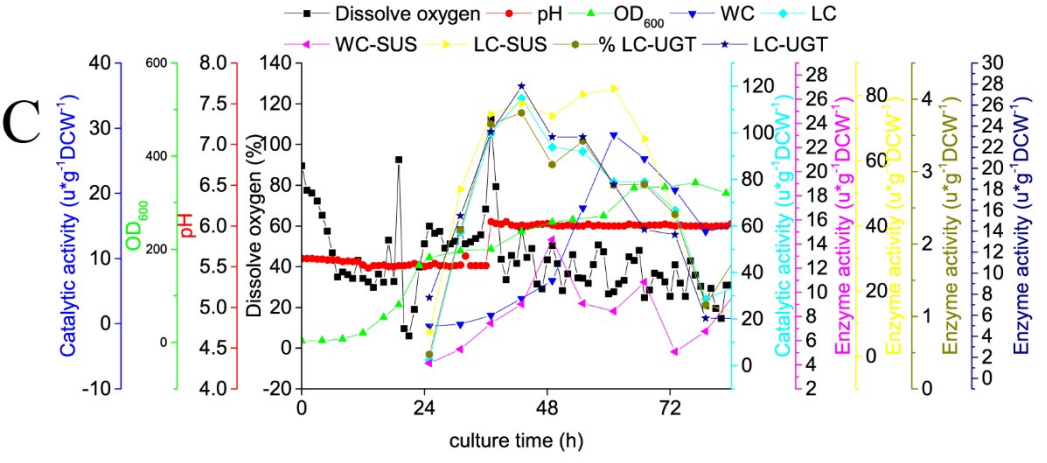
	Initial OD_{600}	Methanol feed rate($g*L^{-1}h^{-1}$)	Temperature (^oC)	pH value
Strategy 1: different initial cell density in stage III				
Pattern 1	40	4	25	6.0
Pattern 2	80	4	25	6.0
Pattern 3	160	4	25	6.0
Pattern 4	320	4	25	6.0
Strategy 2: different methanol feed rate in stage III				
Pattern 5	80	1	25	6.0
Pattern 6	80	2	25	6.0
Pattern 7	80	3	25	6.0
Pattern 8	80	4	25	6.0

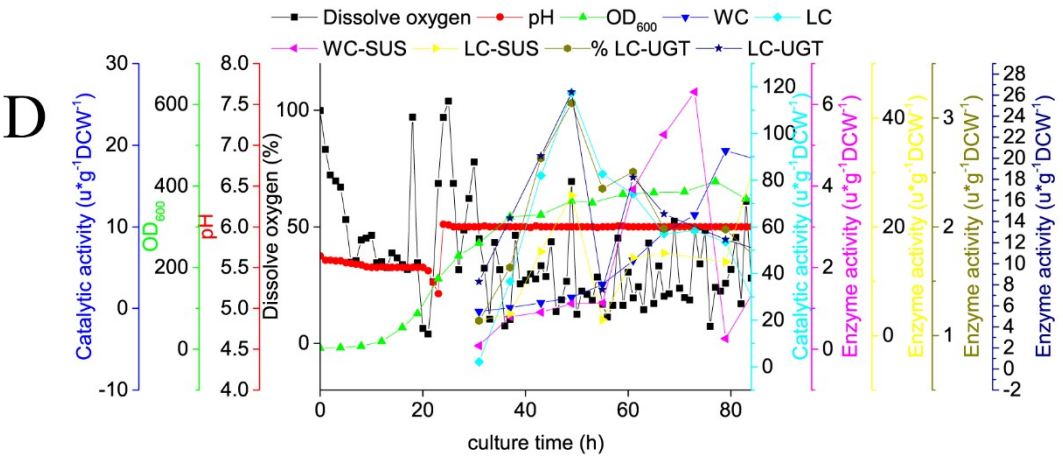
Supplementary Table 5. Reaction system and conditions.

	Sucrose synthase (SUS)	UDP-Glycosyltransferase (UGTs)	Coupling reaction
100 mM PBS (pH)7.0	100 μL	100 μL	100 μL
1 M sucrose	10 μL	/	10 μL
50 mM UDP	8 μL	/	4 μL
50 mM UDPG	/	8 μL	/
100 mM $MgCl_2$	6 μL	6 μL	3 μL
40 g/L stevioside (ST)	/	50 μL	50 μL
Resuspended cell / Cell lysates + ddH2O	74 μL	36 μL	33 μL
Reaction time	20 min	20 min	6 h
Reaction temperature	43 °C	43 °C	43 °C

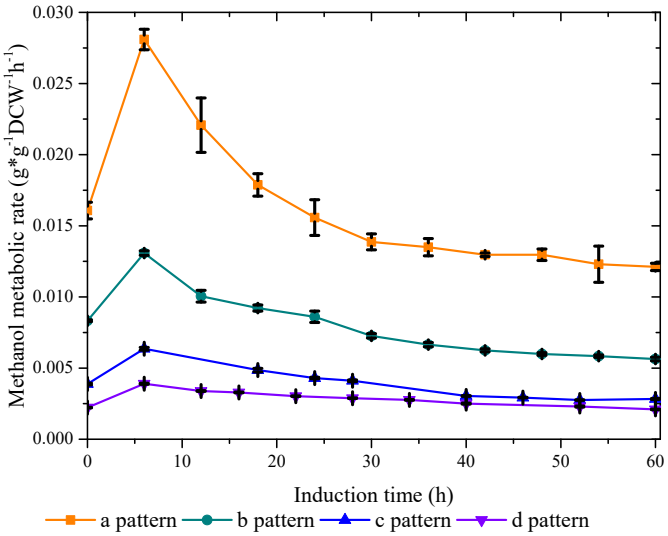

A
Dissolve oxygen
pH
OD600
WC
LC
WC-SUS
LC-SUS
% LC-UGT
LC-UGT
Catalytic activity (u*g-1DCW-1)
OD600
pH
Dissolve oxygen (%)
Enzyme activity (u*g-1DCW-1)
culture time (h)


B
Dissolve oxygen
pH
OD600
WC
LC
WC-SUS
LC-SUS
% LC-UGT
LC-UGT
Catalytic activity (u*g-1DCW-1)
OD600
pH
Dissolve oxygen (%)
Enzyme activity (u*g-1DCW-1)
culture time (h)

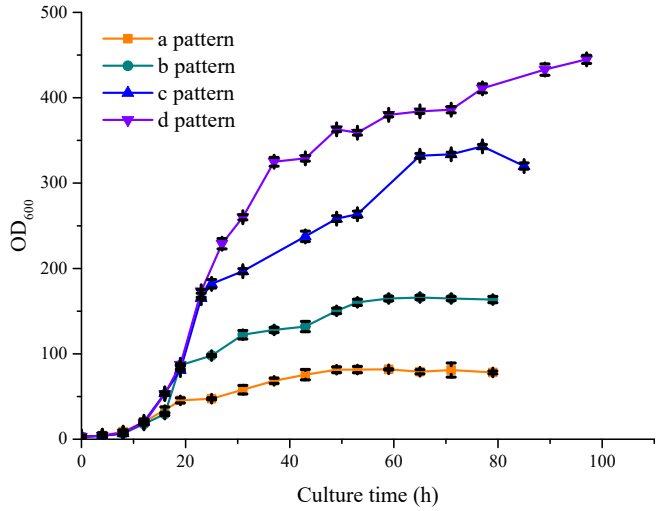

C
Dissolve oxygen
pH
OD600
WC
LC
WC-SUS
LC-SUS
% LC-UGT
LC-UGT
Catalytic activity (u*g-1DCW-1)
OD600
pH
Dissolve oxygen (%)
Enzyme activity (u*g-1DCW-1)
culture time (h)


D
Dissolve oxygen
pH
OD600
WC
LC
WC-SUS
LC-SUS
% LC-UGT
LC-UGT
Catalytic activity (u*g-1DCW-1)
OD600
pH
Dissolve oxygen (%)
Enzyme activity (u*g-1DCW-1)
culture time (h)

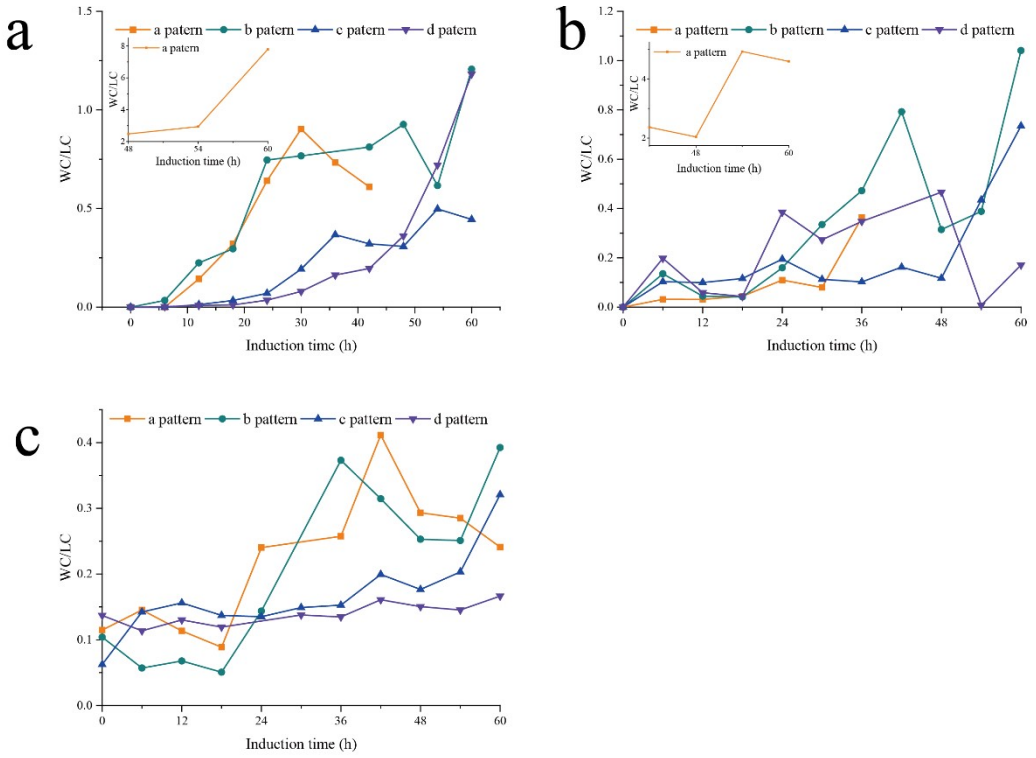
Supplementary Fig. 1 Fermentation process under four different fermentation strategies. A a pattern B a pattern C c pattern D d pattern.



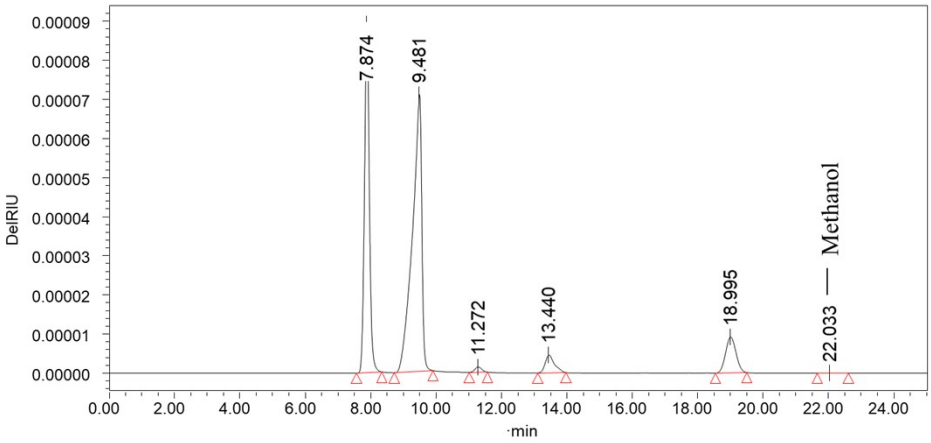
Supplementary Fig. 2 The methanol feed rate of four fermentation strategies.



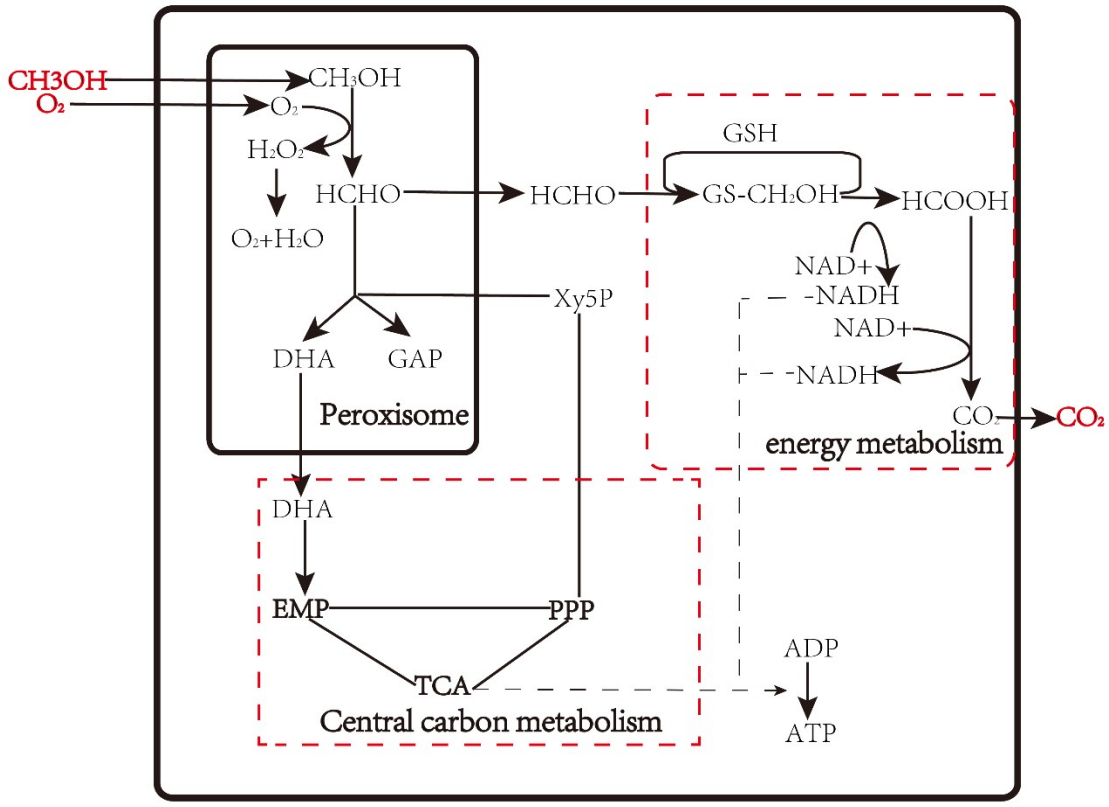
Supplementary Fig. 3 Growth curve of four fermentation strategies.



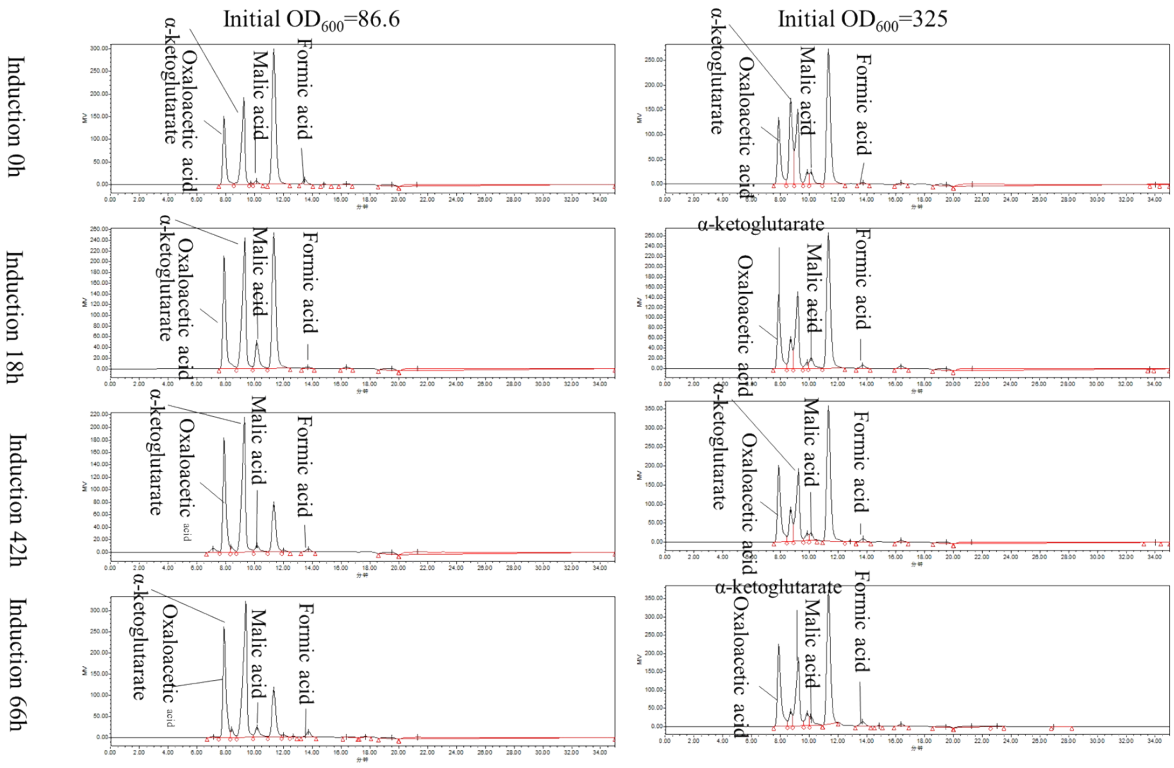
Supplementary Fig. 4 The activity ratio of whole-cell to lyseicell. **a** double enzyme. **b** SUS. **c** UGTs.



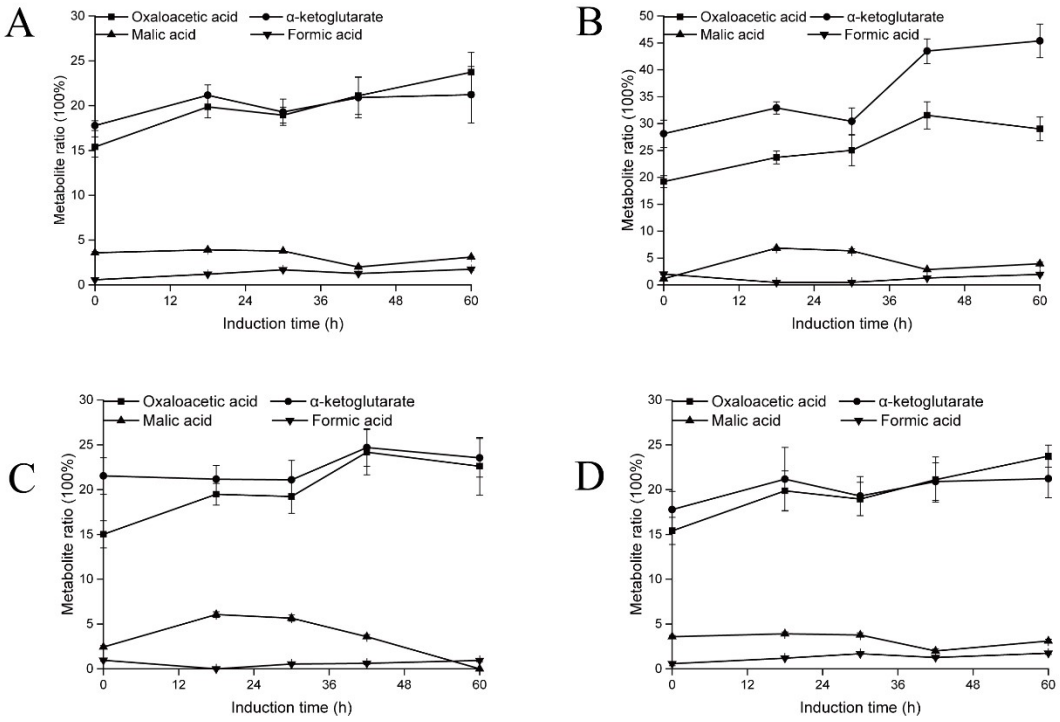
Supplementary Fig. 5 Fingerprint map of fermentation broth



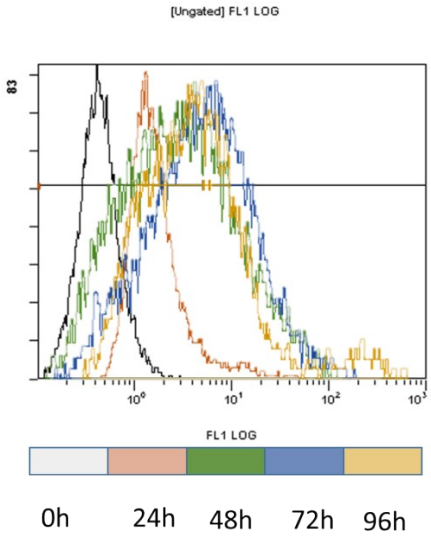
Supplementary Fig. 6 The methanol metabolism network



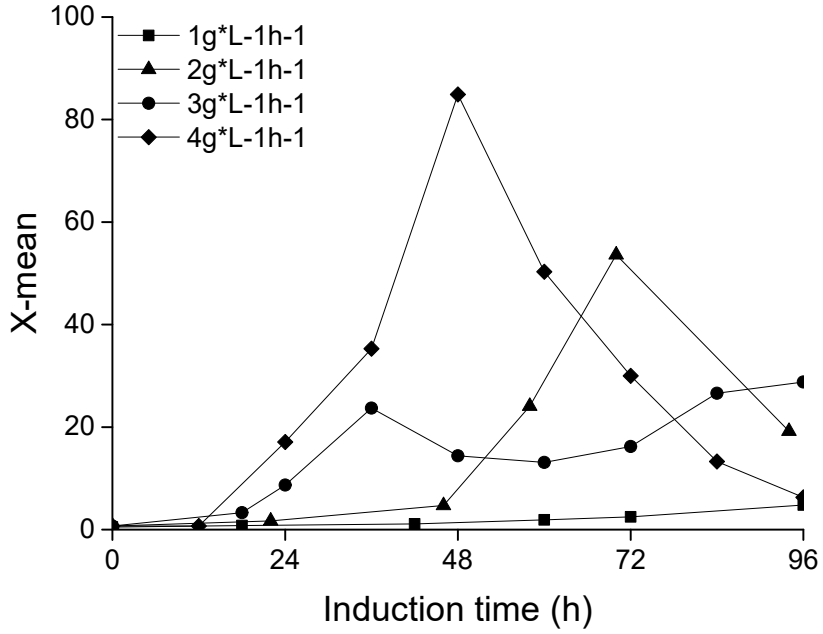
Supplementary Fig. 7 The metabolic fingerprint of Intracellular metabolites



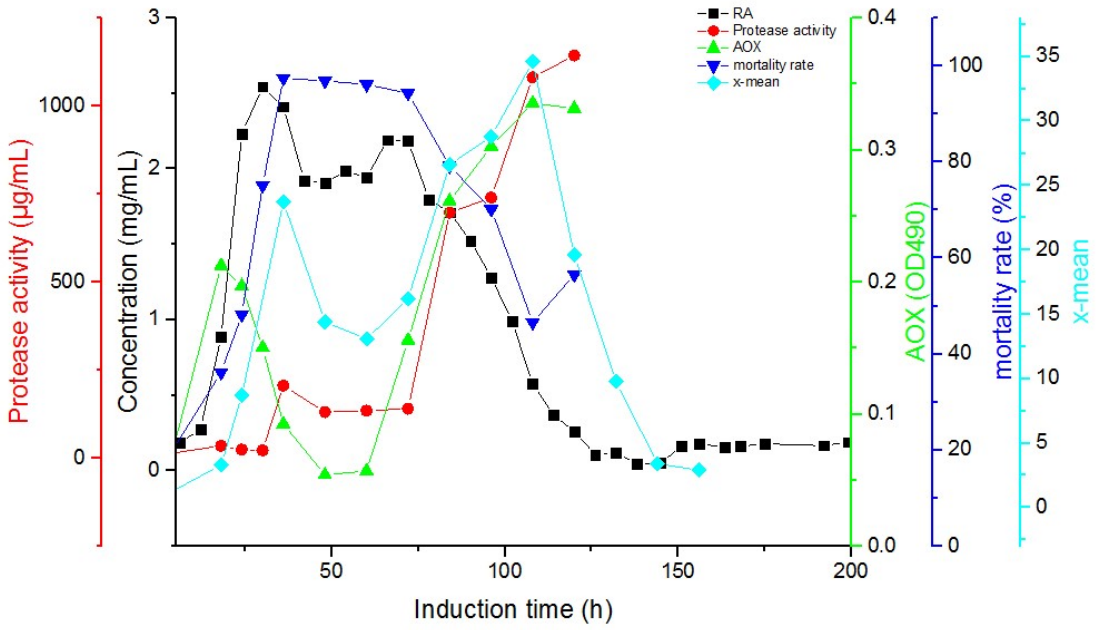
Supplementary Fig. 8 The trend of intracellular metabolites under fermentation strategies. A a pattern B a pattern C c pattern D d pattern.



Supplementary Fig. 9 Flow cytometry for UGT surface display



Supplementary Fig. 10 The surface display of UGT in different methanol feed rate.



Supplementary Fig. 11 The aox activity, protease activity, PI dyeing, UGT surface display and RA yield. 3gL^{-1}h^{-1} to feed methanol.

Supplementary Note 1. Intracellular metabolite analysis under different initial cell densities of induction

According to Celik et al[1], the metabolism of methanol by *Pichia pastoris* occurs by both central carbon metabolism and energy metabolism(**Supplementary Fig. 5**). When central carbon metabolism is greater, it leads to greater protein production [1, 2], and less oxygen consumption. The formic acid was measured to characterize energy metabolism and oxaloacetic acid, α-ketoglutarate, and malic acid synthesis was used to characterize carbon metabolism.

When high catalytic activities occurring, the dissolved oxygen (DO) was rising (**Supplementary Fig. 1**), and metabolic flow migration may be occurring. To verify this hypothesis, intracellular metabolites were analyzed (**Supplementary Fig. 7 and 8**). There was a significant difference in intracellular metabolites that the metabolic flow migrates towards carbon metabolism when initial cell densities of induction change from high (**c** and **d** patterns) to low (**a** and **b** patterns). And the intracellular metabolites species of **c** and **d** patterns were more diverse than **a** and **b** patterns (**Supplementary Fig. 7**). From the whole fermentation process, the four patterns exhibited similar variation trends to metabolism. As fermentation progressed, the formic acid content was not a significant difference, the results indicating that methanol metabolism was not affected, which was consistent with methanol accumulation not being detected in the fermentation broth (**Supplementary Fig. 5**). The oxaloacetic acid, α-ketoglutarate, and malic acid gradually

increased from induction 0 h to 18 h indicated that carbon metabolism gradually increasing which was a significant characteristic of protein accumulation. From 18h to 30h, oxaloacetic acid, α-ketoglutarate, and malic acid gradually decreased. At this stage, the PI staining cells increased and the cells grew slower for cell burden caused the metabolic slowdown of the cells. After induction 30 h, oxaloacetic acid and α-ketoglutarate gradually increased, and malic acid gradually decreased which could be that the self-help mechanism of the cell was at work. The stronger central carbon metabolism would increase the number of cellular functional elements, help cells to relieve the burden.

[1] E. Celik, P. Calik, S.G. Oliver, Metabolic flux analysis for recombinant protein production by Pichia pastoris using dual carbon sources: Effects of methanol feeding rate, Biotechnol Bioeng 105(2) (2010) 317-29. https://doi.org/10.1002/bit.22543.

[2] J. Nocon, M. Steiger, T. Mairinger, J. Hohlweg, H. Rußmayer, S. Hann, B. Gasser, D. Mattanovich, Increasing pentose phosphate pathway flux enhances recombinant protein production in Pichia pastoris, Applied Microbiology and Biotechnology 100(13) (2016) 5955-5963. https://doi.org/10.1007/s00253-016-7363-5.