Supplementary Information for

One-pot efficient biosynthesis of (3*R*)-acetoin from pyruvate by two-enzyme cascade

Zhenzhen Cui, †[a] Yufeng Mao, †[b] Yujiao Zhao, [a] Meiyu Zheng, [a] Zhiwen Wang, *[a] Hongwu Ma, * [b] Tao Chen *[a]

[a] Frontier Science Center for Synthetic Biology and Key Laboratory of Systems Bioengineering (Ministry of Education); SynBio Research Platform, Collaborative Innovation Center of Chemical Science and Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China.

[b] Key Laboratory of Systems Microbial Biotechnology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China.

* Corresponding author: zww@tju.edu.cn, ma_hw@tib.cas.cn and chentao@tju.edu.cn

† These authors contributed equally to this work.

Table S1 Plasmids and enzymes used in this study.

Plasmids	Enzyme	Gene	NCBI-ProteinID	Sources
pET28a-*alsS*$_{bsu}$	bsu-ALS	*alsS*$_{bsu}$	NP_391482	*Bacillus subtilis* 168
pET28a-*alsS*$_{enc}$	enc-ALS	*alsS*$_{enc}$	YP_003613611	*Enterobacter cloacae* ATCC 13047
pET28a-*alsS*$_{stc}$	stc-ALS	*alsS*$_{stc}$	AAV62507	*Streptococcus thermophiles* CNRZ1066
pET28a-*alsD*$_{bsu}$	bsu-ALDC	*alsD*$_{bsu}$	NP_391481	*Bacillus subtilis*168
pET28a-*alsD*$_{llx}$	llx-ALDC	*alsD*$_{llx}$	AII12721	*Lactococcus lactis* NCDO 2118
pET28a-*alsD*$_{stc}$	stc-ALDC	*alsD*$_{stc}$	AAV62508	*Streptococcus thermophilus* CNRZ1066
pET28a-*alsD*$_{enc}$	enc-ALDC	*alsD*$_{enc}$	YP_003613612	*Enterobacter cloacae* ATCC 13047
pET28a-*alsD*$_{glo}$	glo-ALDC	*alsD*$_{glo}$	ACD94444	*Geobacter lovleyi* SZ

Table S2 The primers used in this study.

primers	Nucleotide sequence
alsS$_{bsu}$-F	GCACGGGATCCATGACAAAAGCAACAAAAGAAC
alsS$_{bsu}$-R	CTGCCTCGAGCTAGAGAGCTTTCGTTTTCATGAG
alsD$_{bsu}$-F	GCACGGAATTCATGAAACGAGAAAGCAACATTCAAG
alsD$_{bsu}$-R	GCTTGCTCGAGTTATTCAGGGCTTCCTTCAGTTG

Table S3 Comparison of five enzymes for acetoin formation.

Enzyme	FAD requirement	Sources	Dominated biological function
PDC (EC Number:4.1.1.1)[1-3]	Yes	Bacteria, fungi, and plants	aldehyde-forming
PDH (EC Number:1.2.4.1)[4-6]	Yes	Bacteria, fungi, and plants and animal	acetyl-transferring
POX (EC Number:1.2.3.3)[7]	Yes	Bacteria	acetyl phosphate-forming
AHAS (EC Number:2.2.1.6)[8]	Yes	Bacteria, fungi, and plants	α-acetolactate and α-Aceto-2-hydroxybutanoate forming for L-valine and L-isoleucine biosynthesis.
ALS (EC Number:2.2.1.6)[9, 10]	No	Bacteria (natural acetoin producer)	α-acetolactate-forming for diacetyl and acetoin synthesis

Table S4 The 66 mesophiles or thermophiles species used in ALS and ALDC screening.

KEGG org code list of 66 mesophiles or thermophiles species	sai, gus, gsk, gme, gur, glo, gbm, geo, gem, geb, gpi, gka, gtn, gth, gte, gwc, afl, stc, stl, ste, stn, stu, stw, sthe, aac, aad, ctx, tco, tex, thx, tmt, tbo, twi, tki, toc, ttm, txy, ttm, tsh, tto, tnr, hor, tfu, ace, btp, tli, tma, tmm, tmi, tmw, tmq, tmx, tpt, trp, tna, tnp, thp, thz, thr, tle, tta, tme, taf, fno, fpe, tro.

Table S5 Gibbs energies of the reactions with different pyruvate conversions.

Initial pyruvate concentration (mM)	Conversion (%)	ΔrG' (kJ/mol)
3,000	1	-91.2±6.6
3,000	99	-34.2±6.6
4,500	1	-90.2±6.6
4,500	99	-33.2±6.6

The website used in calculation of Gibbs energies is http://equilibrator.weizmann.ac.il/.
Conversion is the proportion of the molar mass of pyruvate converted to acetoin to initial pyruvate, for example, 1% conversion of 3000 mM pyruvate is the reaction containing the 2,970 mM pyruvate, 55 M (default value) H_2O, 15 mM acetoin and 30 mM CO_2.
ΔrG' is the change in Gibbs free energy with the change of substrate or product concentration due to a chemical reaction at pH 7.0 and ionic strength of 0.1 M.

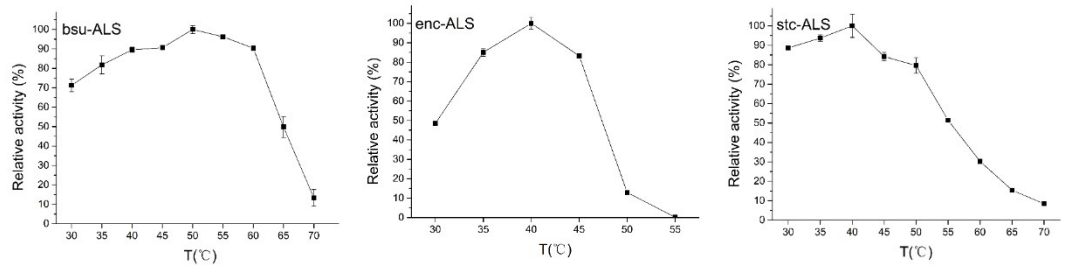

Fig. S1 The effect of different temperatures on ALS activity.

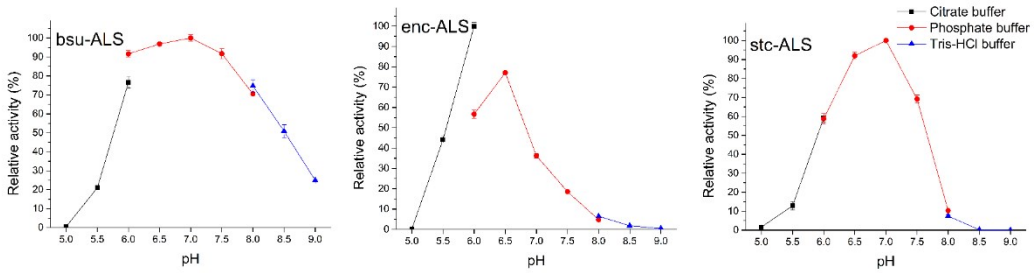

Fig. S2 The effect of different pH on ALS activity. The buffer ranges of citrate buffer, phosphate buffer and Tris-HCl buffer are 3.0-6.6, 5.5-8.5 and 7.1-9.0 respectively.

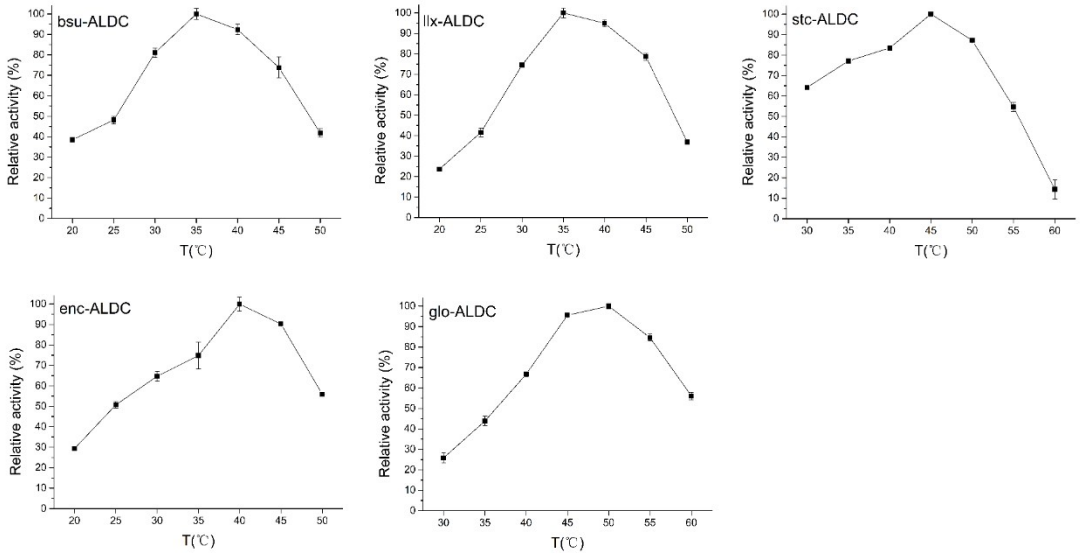

Fig. S3 The effect of different temperatures on ALDC activity.

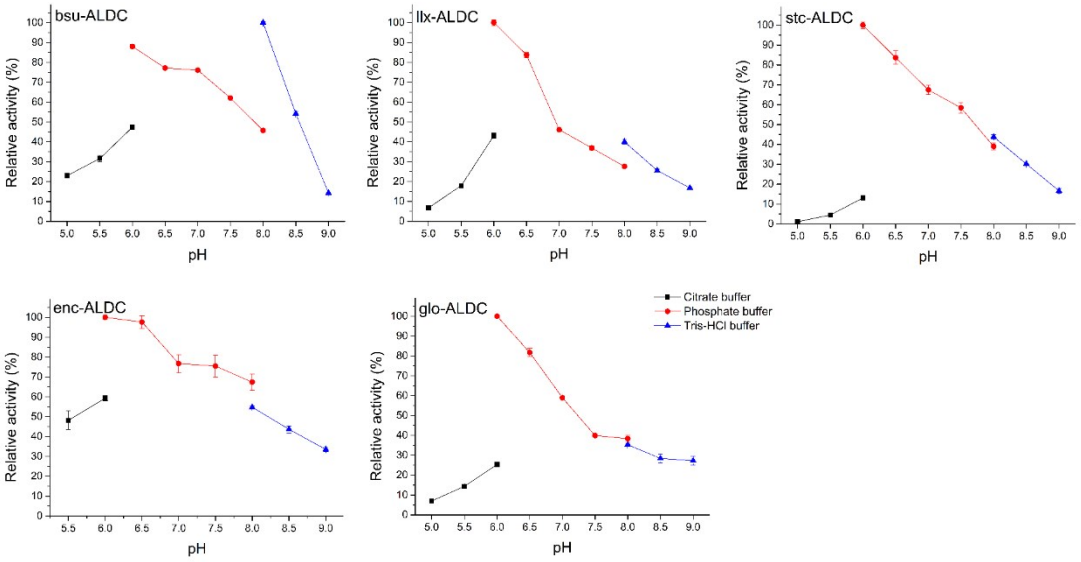

Fig. S4 The effect of different pH on ALDC activity. The buffer ranges of citrate buffer, phosphate buffer and Tris-HCl buffer are 3.0-6.6, 5.5-8.5 and 7.1-9.0 respectively.

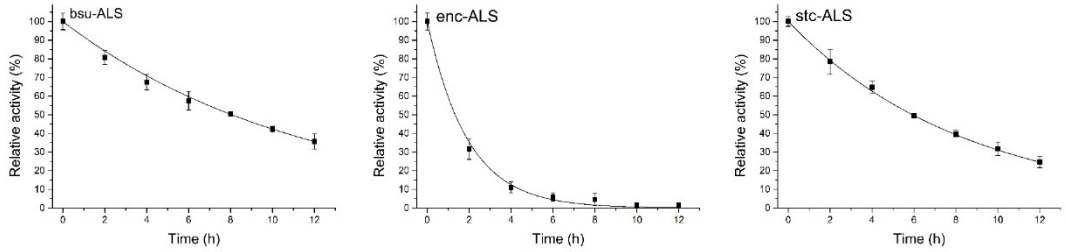

Fig. S5 The stability of ALS at 35 °C, pH 7.0.

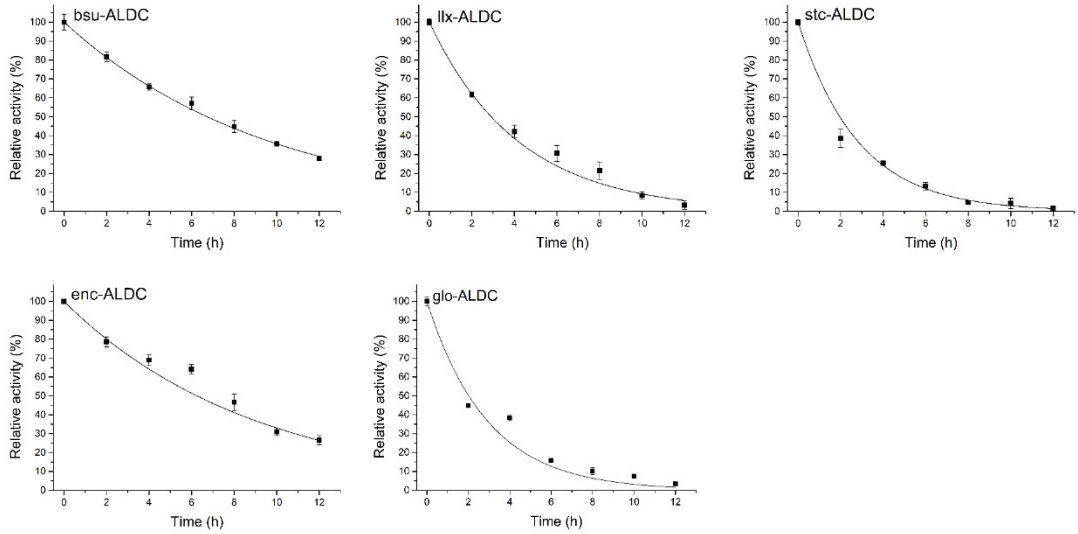

Fig. S6 The stability of ALDC at 35 °C, pH 6.5.

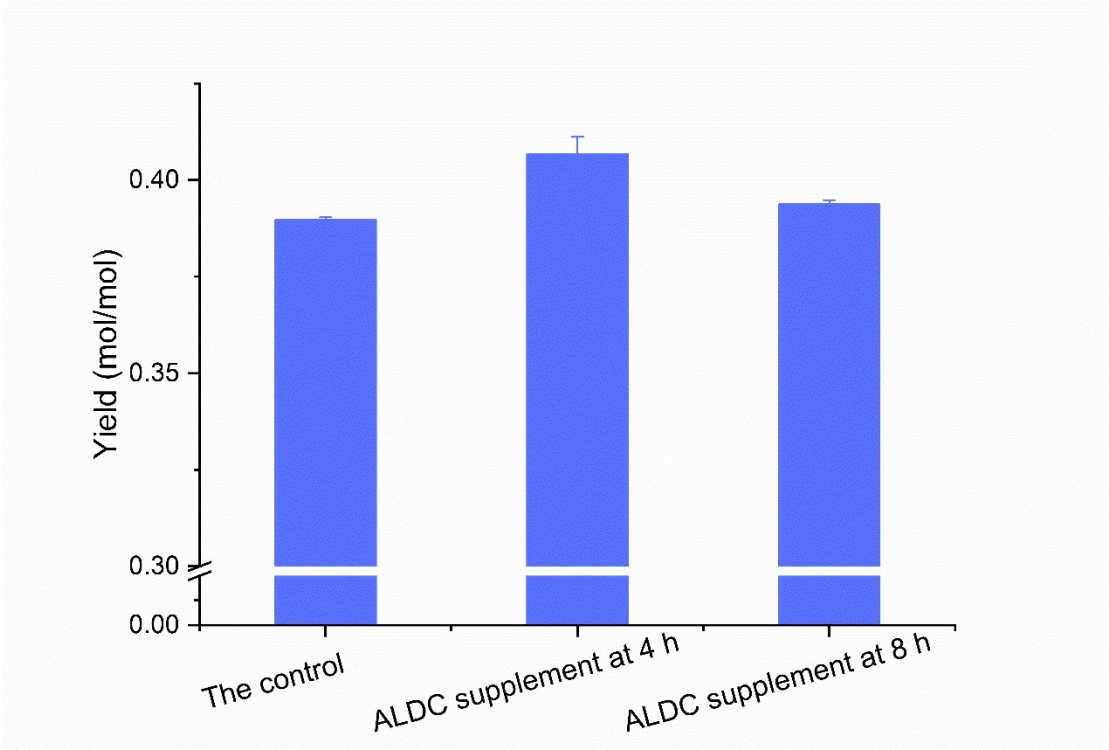

Fig. S7 The yield of (3*R*)-acetoin without or with supplement of bsu-ALDC at 4 h or 8 h.

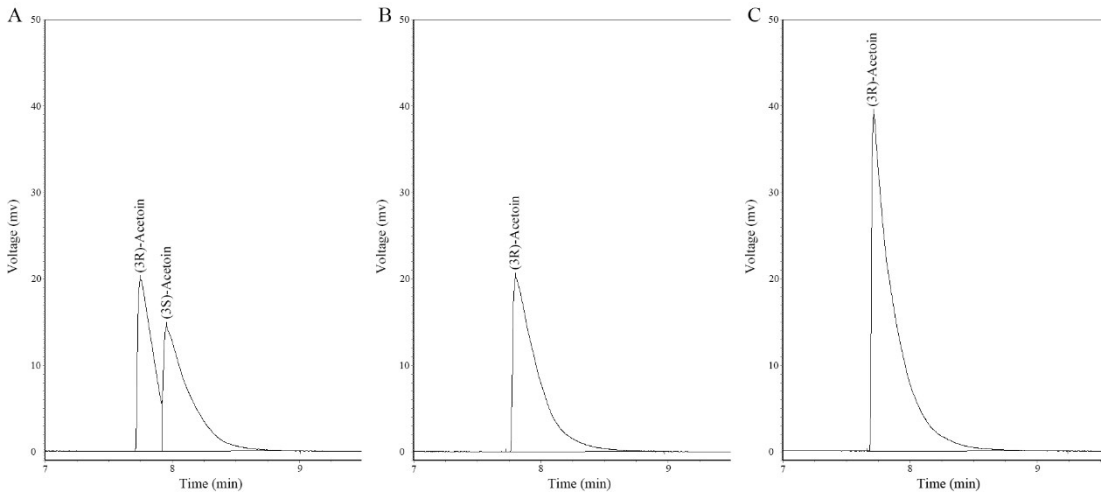

Figure S8. Identification of (3*R*)-acetoin enantiomeric excess by GC-FID. A, (3*R*)-Acetoin and (3*S*)-acetoin enantiomeric standards, respectively. B, (3*R*)-Acetoin produced by enzymatic system in 20 mL buffer containing 2.83 M pyruvate, 10 mM $MgCl_2$, 0.2 mM TPP, 3.6 U/mL bsu-ALS and 3.6 U/mL bsu-ALDC at 35 ℃, pH 6.5. C, (3*R*)-Acetoin produced by enzymatic system in 20 mL buffer containing 4.492 M pyruvate, 10 mM $MgCl_2$, 0.2 mM TPP, .5.4 U/mL bsu-ALS and 5.4 U/mL bsu-ALDC at 35 ℃, pH 6.5.

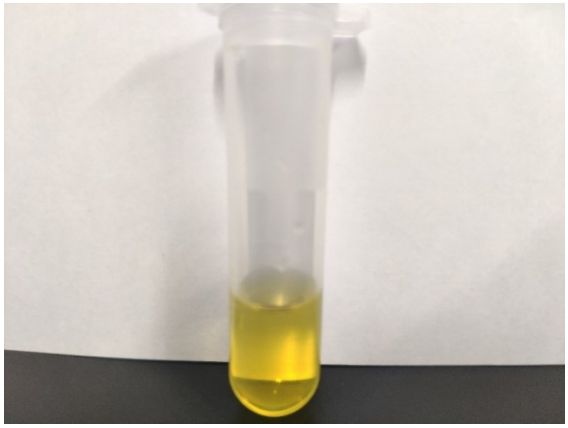

Fig. S9 The image of acetoin liquid after rotary evaporation.

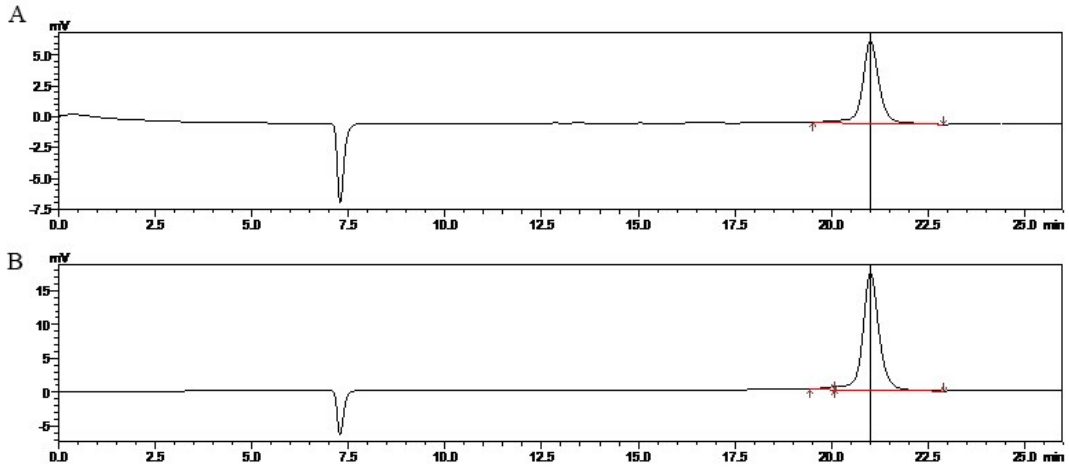

Fig. S10 The purity identification of purified acetoin by HPLC. A, Acetoin standard purchased from Tokyo Chemical Industry. The surface areas and retention time of acetoin standard (2.275 g/L) was 253957 mV*min and 21.020 min respectively. B, Acetoin obtained in our work. The surface areas and retention time of acetoin solution (4.669 g/L) was 512012 mV*min (4.587 g/L) and 21.012 min respectively.

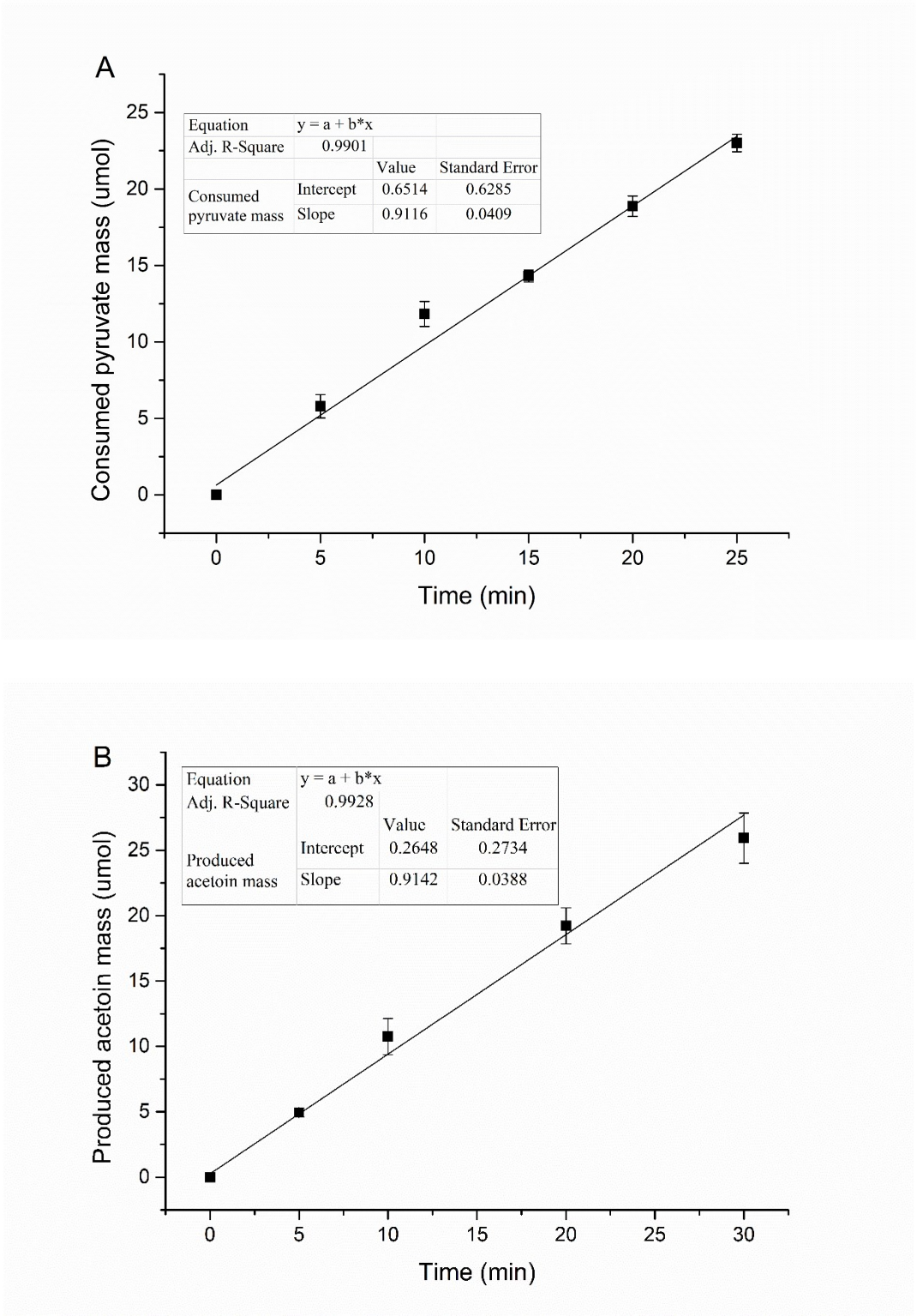

Fig. S11 The activities of ALS and ALDC measured by HPLC methods. A, Time profile of consumed pyruvate mass in ALS assayed system. B, Time profile of produced acetoin mass in ALDC assayed system.

Reference

1. Y. G. Wu, A. K. Chang, P. F. Nixon, W. Li and R. G. Duggleby, *Eur. J. Biochem.*, 2000, **267**, 6493-6500.
2. E. A. Sergienko and F. Jordan, *Biochemistry*, 2001, **40**, 7369-7381.
3. M. A. Gabriel, H. Jabara and U. A. al-Khalidi, *The Biochemical journal*, 1971, **124**, 793-800.
4. J. D. Hillman, S. W. Andrews and A. L. Dzuback, *Infect. Immun.*, 1987, **55**, 1399-1402.
5. J. A. Montgomery, M. Jetté, S. Huot and C. Des Rosiers, *The Biochemical journal*, 1993, **294 (Pt 3)**, 727-733.
6. L. G. Baggetto and A. L. Lehninger, *J. Biol. Chem.*, 1987, **262**, 9535-9541.
7. B. L. Bertagnolli and L. P. Hager, *Arch. Biochem. Biophys.*, 1993, **300**, 364-371.
8. Y. Y. Chang and J. E. Cronan, Jr., *J. Bacteriol.*, 1988, **170**, 3937-3945.
9. S. S. Pang, R. G. Duggleby, R. L. Schowen and L. W. Guddat, *J. Biol. Chem.*, 2004, **279**, 2242-2253.
10. F. C. Störmer, *J. Biol. Chem.*, 1967, **242**, 1756-1759.