Electronic Supplementary Material (ESI) for Green Chemistry.

Highly efficient biosynthesis of 2,4-dihydroxybutyric acid by a methanol assimilation pathway in engineered *Escherichia coli*

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^{1}H-NMR and ^{13}C-NMR analysis of the 2,4-DHB

^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker NMR spectrometers (600 MHz, in D_2O). ^{1}H-NMR and ^{13}C-NMR chemical shifts (δC) are quoted in parts per million (ppm) downfield from trimethyl silane (TMS), and coupling constants (J) are quoted in Hertz (*Hz*).

LC-MS analysis of the 2,4-DHB

The cell and supernatant were separated by centrifugation at 13,000 rpm for 30 min, and 1 μL aliquot of the supernatant was injected into an LC-MS system. The HPLC was equipped with a Thermo Aclaim Organic Acid HPLC column (4 mm x 250 mm, 5 μm) and eluted with a flow rate of 0.2 mL/min. The mobile phase A consisted of 0.1% of formic acid in water, and the mobile phase B was acetonitrile. The gradient elution was performed as described below:0-4 min, 0%B; 4-10 min, 30%B; 10-12 min, 80%B; 12-17 min, 80%; 17-25 min, 0%B. A Compact Q-TOF mass spectrometry (Bruker Daltonics, Billerica, USA) with an ESI source in negative ion mode was used for MS analysis.

GC-MS analysis of the α-hydroxy-γ-butyrolactone

The aqueous solution of 2,4-DHB was adjusted to pH 2-12 with 10 M potassium hydroxide and hydrochloric acid, respectively. And the percentages of 2,4-DHB and α-hydroxy-γ-butyrolactone were detected using GC-MS. The α-hydroxy-γ-butyrolactone was assayed as follows: An equal amount of ethyl acetate was added to the aqueous solution after pH adjustment, vortexed briefly for 2 min, and centrifuged to separate the phases. 1 μL was injected with using a split ratio 40:1 on an SH-Rtx-5MS Cap. Column (30 m, 0.25 mm ID, 0.25 μm) using helium as the carrier gas. The inlet temperature and flow rate were set at 280 ℃ and 3.0 mL/min, respectively. The oven temperature gradient program was set as follows: initially held at 70 ℃ for 2 min, raised by 10 ℃/min to 250 ℃, hold for 5 min. The total run time was 25 min. The MS conditions for identification of α-hydroxy-γ-butyrolactone was as follows: for Q1 scan mode, 30-500 m/z mass-range. Quantification was based on the peak area ratios of the different compounds to standard chemicals.

Table S1 Strains and plasmids used in this study.

Strain and plasmid	Descriptions	Sources
Strains		
E. coli DH5α	strain used for general cloning	Laboratory stock
E. coli BL21(DE3)	Strain used for constructing engineering bacteria and protein expression	Laboratory stock
E. coli W3110	Strain used for constructing engineering bacteria and protein expression	Laboratory stock
JW0855	*E. coli K-12 poxB*::*kan*	Keio collection
JW0886	*E. coli K-12 pflB*::*kan*	Keio collection
JW1228	*E. coli K-12 adhE*::*kan*	Keio collection
JW2294	*E. coli K-12 pta*::*kan*	Keio collection
D1	*E. coli* W3110 *poxB*::*kan*	This study
D2	*E. coli* W3110 Δ*poxB*	This study
D3	*E. coli* W3110 Δ*poxBpflB*::*kan*	This study
D4	*E. coli* W3110 Δ*poxB*Δ*pflB*	This study
D5	*E. coli* W3110 Δ*poxB*Δ*pflB*Δ*adhE*::*kan*	This study
D6	*E. coli* W3110 Δ*poxB*Δ*pflB*Δ*adhE*	This study
JW0348	*E. coli K-12 frmR*::*kan*	Keio collection
JW0347	*E. coli K-12 frmA*::*kan*	Keio collection
JW0346	*E. coli K-12 frmB*::*kan*	Keio collection
D7	*E. coli* W3110 Δ*poxB*Δ*pflB*Δ*adhE*Δ*frmRAB*::*kan*	This study
D8	*E. coli* W3110 Δ*poxB*Δ*pflB*Δ*adhE*Δ*frmRAB*	This study
DHB1	*E. coli* W3110 harboring pETDuet-lac-*khb-par*	This study
DHB2	*E. coli* W3110 harboring pETDuet-lac-*khb-pgcr*	This study
DHB3	*E. coli* W3110 harboring pETDuet-lac-*khb-cpar4*	This study
DHB4	*E. coli* W3110 harboring pETDuet-lac-*khb*-l*dh*	This study
DHB5	*E. coli* W3110 harboring pETDuet-lac-*khb-2ldh*	This study
DHB6	*E. coli* W3110 harboring pETDuet-lac-*khb*-m*dh*	This study
DHB7	*E. coli* W3110Δ*poxB* harboring pETDuet-lac-*khb*-l*dh*	This study
DHB8	*E. coli* W3110Δ*poxB*Δ*pflB* harboring pETDuet-lac-*khb*-l*dh*	This study
DHB9	*E. coli* W3110Δ*poxB*Δ*pflB*Δ*adhE* harboring pETDuet-lac-*khb*-l*dh*	This study
DHB10	*E. coli* W3110Δ*poxB*Δ*pflB*Δ*adhE* harboring pETDuet-lac-$mdh2_{Bm}$-*khb*-l*dh*	This study
DHB11	*E. coli* W3110Δ*poxB*Δ*pflB*Δ*adhE* harboring pETDuet-lac-mdh_{Cn}-*khb*-l*dh*	This study
DHB12	*E. coli* W3110Δ*poxB*Δ*pflB*Δ*adhE* harboring pETDuet-lac-mdh_{cn}(G48F)-*khb*-l*dh*	This study
DHB13	*E. coli* W3110Δ*poxB*Δ*pflB*Δ*adhE*ΔfrmRAB harboring pETDuet-lac-mdh_{cn}(G48F)-*khb*-l*dh*	This study

DHB14	*E. coli* BL21(DE3) harboring pET28a (+)-$mdh2_{Bm}$ from *B. methanolicus* MGA3	This study
DHB15	*E. coli* BL21(DE3) harboring pET28a (+)-mdh_{cn} from *Cupriavidus necator*	This study
DHB16	*E. coli* BL21(DE3) harboring pET28a (+)-mdh_{Cn}(G48F)	This study
DHB17	*E. coli* BL21(DE3) harboring pET28a (+)-mdh_{Cn}(M52F)	This study
DHB18	*E. coli* BL21(DE3) harboring pET28a (+)-mdh_{Cn}(C369N)	This study
Plasmids		
pCP20	Amp^R Cm, FLP recombinase expression	Laboratory stock
pKD46	Amp^R, λ Red recombinase expression	Laboratory stock
pETDuet-1	*E. coli* expression vector, Amp^R, T7 promoter	Laboratory stock
pETDuet-lac	*E. coli* expression vector, Amp^R, lac_{1-6} promoter	This study
pETDuet-lac-*khb*	pETDuet-lac, Amp^R, *khb* expression, lac promoter	This study
pETDuet-lac-*khb-par*	pETDuet-lac-*khb,* Amp^R, *par* expression, lac promoter	This study
pETDuet-lac-*khb-pgcr*	pETDuet-lac-*khb*, Amp^R, *pgcr* expression, lac promoter	This study
pETDuet-lac-*khb-cpar4*	pETDuet-lac-*khb*, Amp^R, *cpar4* expression, lac promoter	This study
pETDuet-lac-*khb-ldh*	pETDuet-lac-*khb*, Amp^R, *ldh* expression, lac promoter	This study
pETDuet-lac-*khb-2ldh*	pETDuet-lac-*khb*, Amp^R, *2ldh* expression, lac promoter	This study
pETDuet-lac-*khb-mdh*	pETDuet-lac-*khb*, Amp^R, *mdh* expression, lac promoter	This study
pETDuet-lac-$mdh2_{Bm}$-*khb-ldh*	pETDuet-lac-*khb-ldh*, Amp^R, $mdh2_{Bm}$, $mdh2_{Bm}$ expression, lac promoter	This study
pET28a(+)	E. coli expression vector, Kan^R, T7 promoter	This study
pET28a-$mdh2_{Bm}$	pET28a(+), Kan^R, $mdh2_{Bm}$ expression, T7 promoter	This study
pET28a-mdh_{Cn}	pET28a(+), Kan^R, mdh_{Cn} expression, T7 promoter	This study
pET28a-mdh_{Cn}(G48F)	pET28a(+)-mdh_{Cn}, Kan^R, $mdh_{Cn/G48F}$ expression, T7 promoter	This study
pET28a-mdh_{Cn}(M52F)	pET28a(+)-mdh_{Cn}, Kan^R, $mdh_{Cn/M52F}$ expression, T7 promoter	This study
pET28a-mdh_{Cn}(C369N)	pET28a(+)-mdh_{Cn}, Kan^R, $mdh_{Cn/C369N}$ expression, T7 promoter	This study
pETDuet-lac-mdh_{Cn}-*khb-ldh*	pETDuet-lac-*khb-ldh*, Amp^R, mdh_{Cn} expression, lac promoter	This study

pETDuet-lac-*mdh*$_{Cn}$(G48F)-*khb-ldh*	pETDuet-lac-*mdh*$_{Cn}$-*khb-ldh*, Amp^{R}, *mdh*$_{Cn}^{G48F}$ expression, lac promoter	This study

Table S2 List of PCR primers sequences used in this study.

Primers	Descriptions (5'-3')	Sources
khb-F	taagaaggagatataccatgggcAAAAACTGGAAAACAAGTGCA	This study
khb-R	gccgagctcgaattcggatccTTACAGCTTAGCGCCTTCTACAGC	This study
par-F	taagaaggagatatacatatgAAAGCGATTCAGTATACCCG	This study
par-R	gccgatatccaattgagatctTTAGCGCGGGGTGCAGCT	This study
pgcr-F	taagaaggagatatacatatgAAAAGCATGATTAACGAAAACA	This study
pgcr-R	gccgatatccaattgagatctTTACGGCGCGCAATAGCC	This study
cpar4-F	taagaaggagatatacatatgAGCGCGCAGCTGAAA	This study
cpar4-R	gccgatatccaattgagatctTTAATCGTTAAAGTTGTTAAAGCCCG	This study
ldh-F	taagaaggagatatacatatgGCAAGTATTACGGATAAGGATC	This study
ldh-R	gccgatatccaattgagatctTTACTGACGAGTTTCGATGTCATTC	This study
2ldh-F	taagaaggagatatacatatgACAAAAATTCTAATGTATACCGTCCG	This study
2ldh-R	gccgatatccaattgagatctTTAGTTGACTTGATGCGCTGACTT	This study
mdh-F	taagaaggagatatacatatgAAAGTCGCAGTCCTCGGCG	This study
mdh-R	gccgatatccaattgagatctTTACTTATTAACGAACTCTTCGCCC	This study
mdh2$_{Bm}$-F	taagaaggagatataccatggGCAAAAACACCCAGTCTGCTTT	This study
mdh2$_{Bm}$-R	CCAGTTTTTCATggtatatctccttTTACATAGCGTTTTT	This study
mdh$_{Cn}$-F	taagaaggagatataccatgggcACCCATCTGAACATTGCGAACC	This study
mdh$_{Cn}$-R	CAGTTTTTCATggtatatctccttTTACATCGCCG	This study
mdh2$_{Bm}$-BamHI	cagcaaatgggtcgcggatccATGAAGAATACTCAGTCAGCTTTCTACA	This study
mdh2$_{Bm}$-SacI	gcaagcttgtcgacggagctcTCACATTGCATTCTTAATAATCTGAATAA	This study
mdh$_{Cn}$-BamHI	cagcaaatgggtcgcggatccATGACCCATCTGAACATTGCG	This study
mdh$_{Cn}$-SacI	gcaagcttgtcgacggagctcTTACATCGCCGCCGCAAA	This study
G48F-F	CGATGCGtttCTGCATAAAATGGGCCTGAGCG	This study
G48F-R	TATGCAGaaaCGCATCGGTCACAATCAGAGCT	This study
M52F-F	CCTGCATAAAtttGGCCTGAGCGAAGTGGTGG	This study
M52F-R	GGCCaaaTTTATGCAGGCCCGCATCGGTCACA	This study
S265R-F	TTTAACAACGCGgcgCTGGGCTATGTGCATGCGA	This study
S265R-R	AGcgcCGCGTTGTTAAACGCCATGCCCGCCAG	This study
C369N-F	AGATGCGaacATGCTGACCAACCCGCGCAAAG	This study
C369N-R	TCAGCATgttCGCATCTTTCTGCGCGTTGCTC	This study
pKD4-Km300-3'	GGTGAGATGACAGGAGATCCT	This study
poxB-F	CCGAAATCGC TGAAGGTTAC	This study
pflB-F	CACCGGAAAA TTTTTCTCAC	This study
adhE-F	CACCGGAAAA TTTTTCTCAC	This study
pta-F	CATATGTTTT GTCAAAATGT	This study
FrmRAB-F	TGATCACCAGCTACCGCTATACC	This study

Table S3 The specific activities of the $Mdh2_{Bm}$ and Mdh_{Cn}.

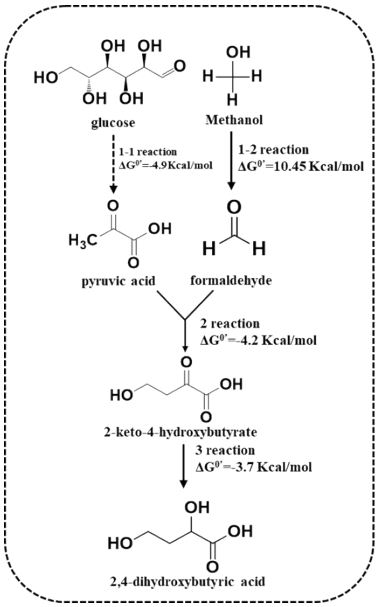
Enzyme	Specific activity (U/mg)
$Mdh2_{Bm}$	0.003
Mdh_{Cn}	0.023

Note：Data are presented as the mean (n=3).

Table S4 The specific activities of the Mdh_{Cn} and its mutants.

Mutation	Specific activity (U/mg)
WT (Mdh_{Cn})	0.023
G48F	0.074
M52F	0.032
C369N	0.046

Note：Data are presented as the mean (n=3).



1-1st equation glucose + 2ADP + 2NAD+ 2Pi ⟶ 2pyruvate + 2ATP + 2NADH+ 2H+ + 2H2O

Formation ΔG0 = -4.9 Kcal/mol

1-2st equation methanol + NAD+ ⟶ formaldehyde + NADH + H+

Formation ΔG0 (Kcal/mol)	-45.15	-529	-30.4	-523.8	-9.5

2st equation pyruvate + formaldehyde ⟶ 2-keto-4-hydroxybutyrate

Formation ΔG0 (Kcal/mol)	-115.2	-30.4	-149.8

3st equation 2-keto-4-hydroxybutyrate + NADH + H+ ⟶ 2,4-dihydroxybutyric acid + NAD+

Formation ΔG0 (Kcal/mol)	-149.8	-523.8	-9.5	-157.8	-529

Fig. S1 Gibbs free energy of the synthetic pathway of 2,4-DHB using the glucose and methanol (or formaldehyde) as co-substrate.

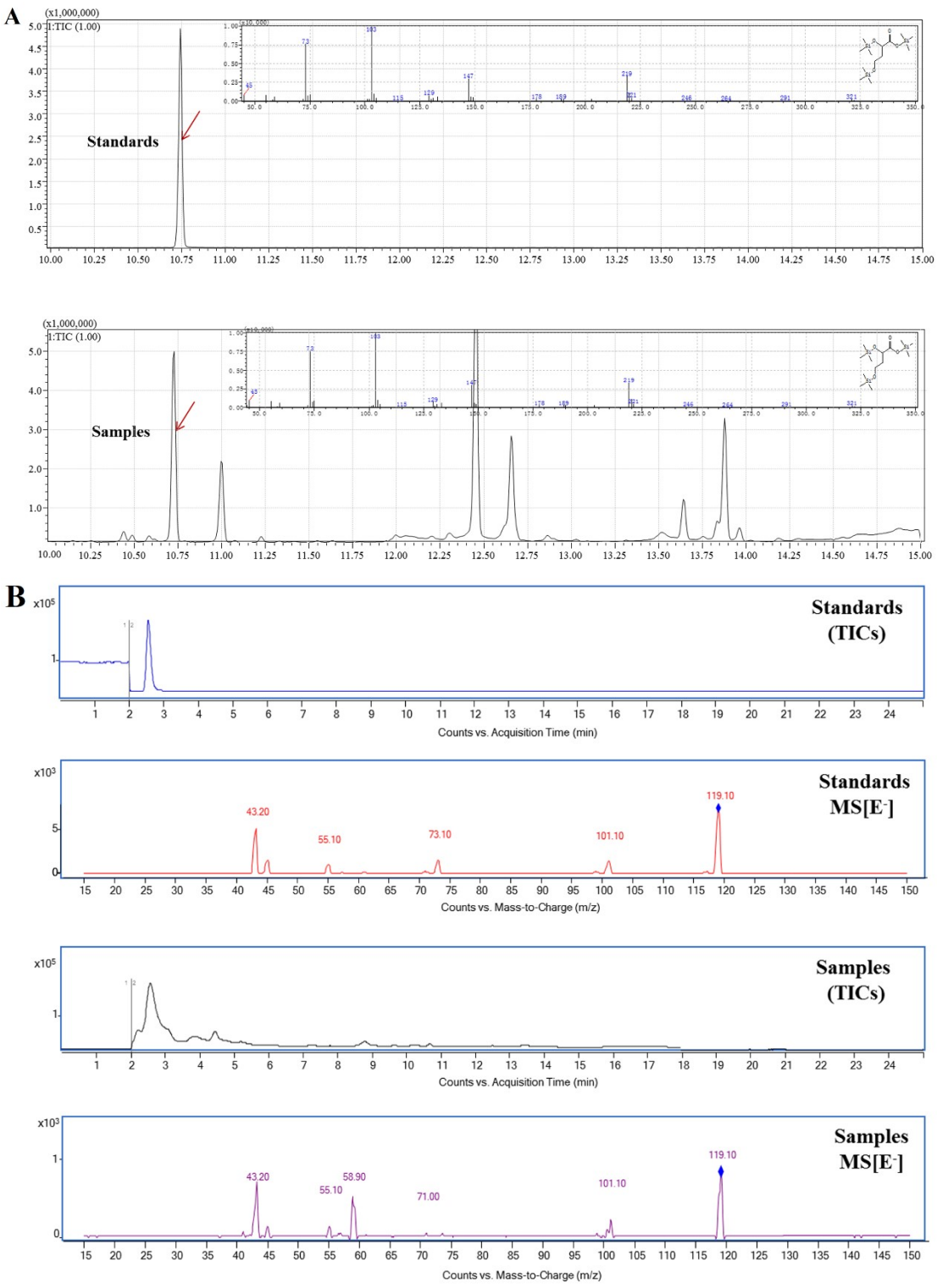
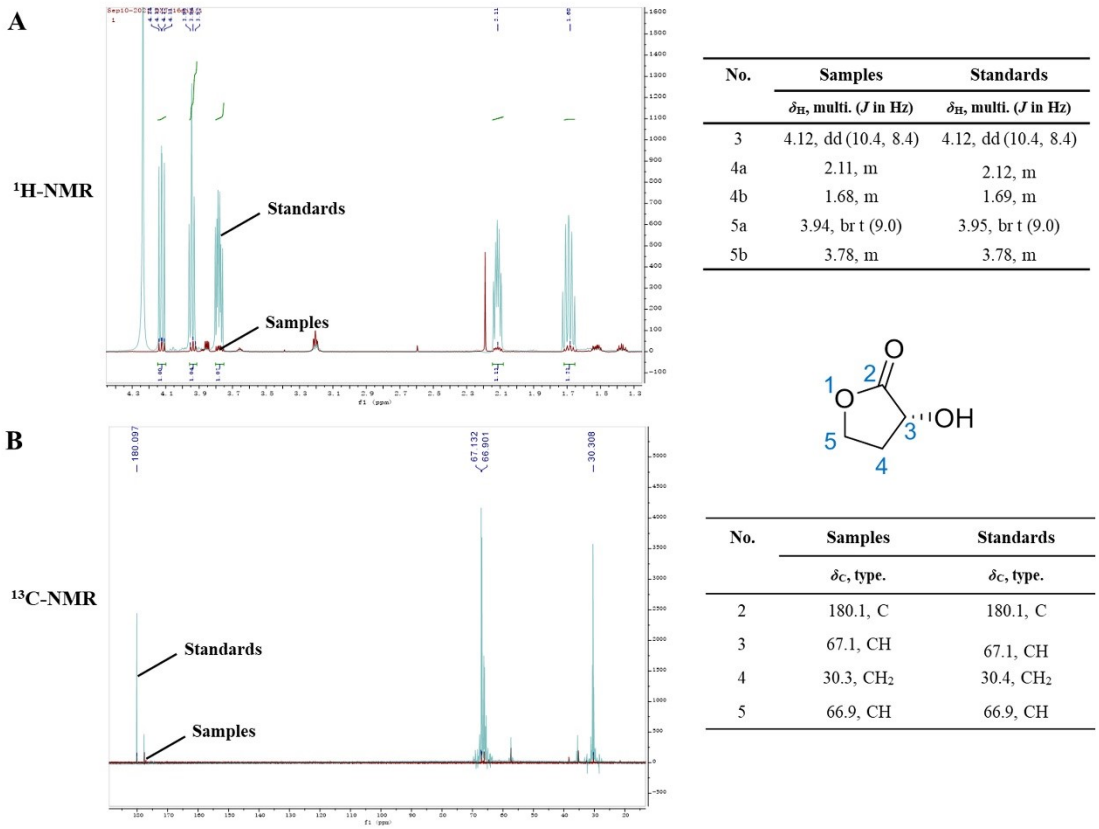


Fig. S2 Verification of 2,4-DHB synthesis from glucose and formaldehyde using GC-MS and LC-MS analysis. (A) GC-MS analysis of standard substance and sample. (B) The total ion chromatograms (TICs) and HRESI-MS (MS[E^-]) spectrum of standard substance and sample.



No.	Samples	Standards
	δ_H, multi. (J in Hz)	δ_H, multi. (J in Hz)
3	4.12, dd (10.4, 8.4)	4.12, dd (10.4, 8.4)
4a	2.11, m	2.12, m
4b	1.68, m	1.69, m
5a	3.94, br t (9.0)	3.95, br t (9.0)
5b	3.78, m	3.78, m

No.	Samples	Standards
	δ_C, type.	δ_C, type.
2	180.1, C	180.1, C
3	67.1, CH	67.1, CH
4	30.3, CH_2	30.4, CH_2
5	66.9, CH	66.9, CH

Fig. S3 Verification of 2,4-DHB synthesis from glucose and formaldehyde using NMR analysis. (A) ^{1}H NMR spectrum of 2,4-DHB standard substance and purified product; (B) ^{13}C NMR spectrum of 2,4-DHB standard substance and purified product.

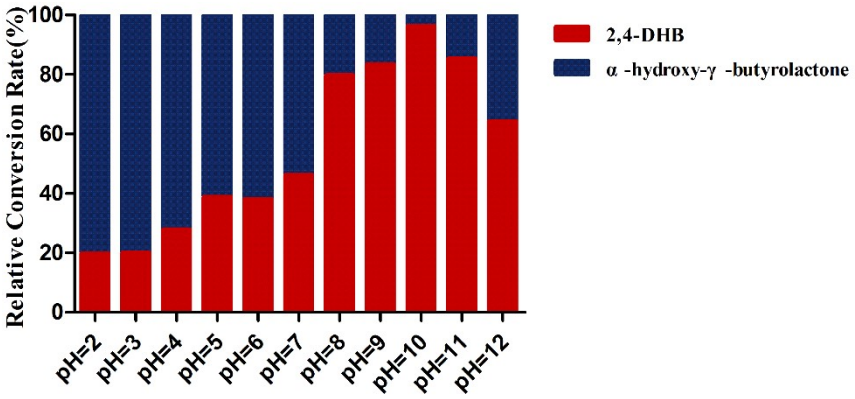


Fig. S4 Interconversion between 2,4-DHB and α-hydroxy-γ-butyrolactone under pH-neutral conditions.

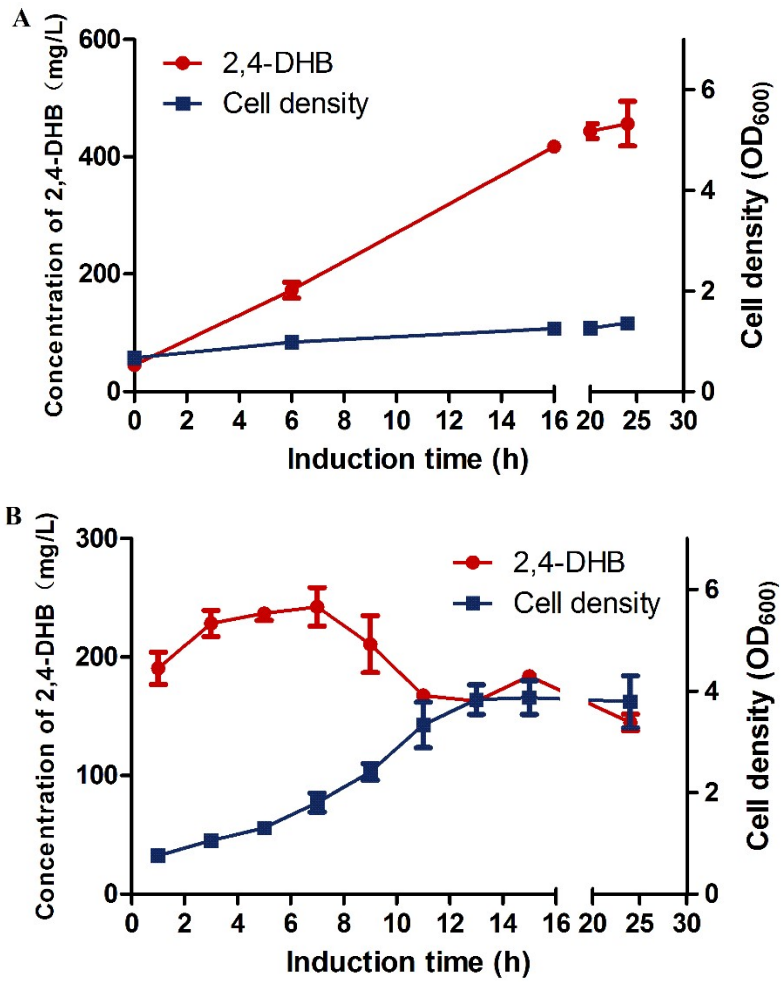


Fig. S5 Time profiles of 2,4-DHB biosynthesis with different substrates. (A) The production of 2,4-DHB from formaldehyde and glucose co-substrate. (B) The production of 2,4-DHB from methanol and glucose co-substrate.