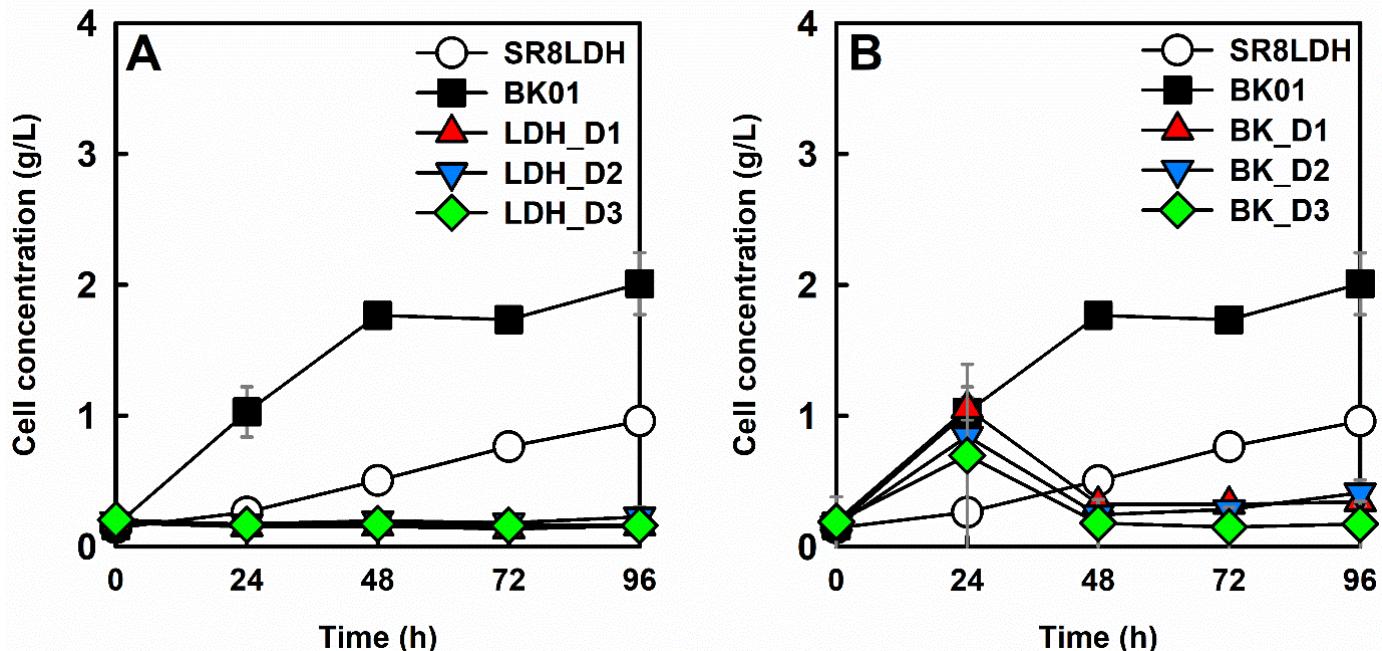


Supplementary Material


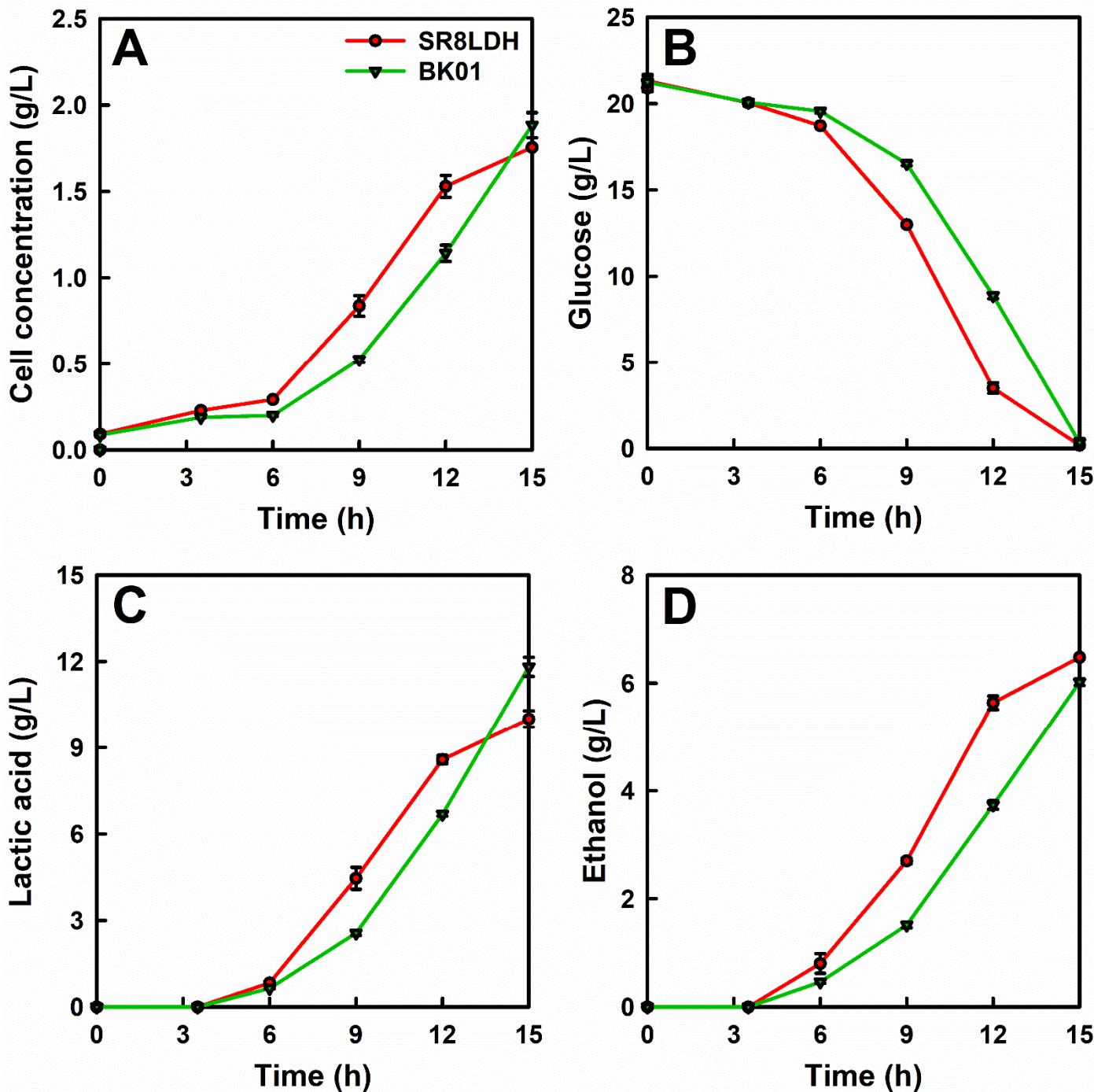


Figure S2. Fermentation profiles of the SR8LDH and BK01 strain in complex medium containing 20 g/L glucose. (A) Cell concentration, (B) glucose consumption, (C) lactic acid production, and (D) ethanol production. The values are the mean of three independent experiments, and the error bars indicate the standard deviations. All experiments were conducted under oxygen-limited conditions (80 rpm), with an initial cell concentration of 0.05 g DCW/L.

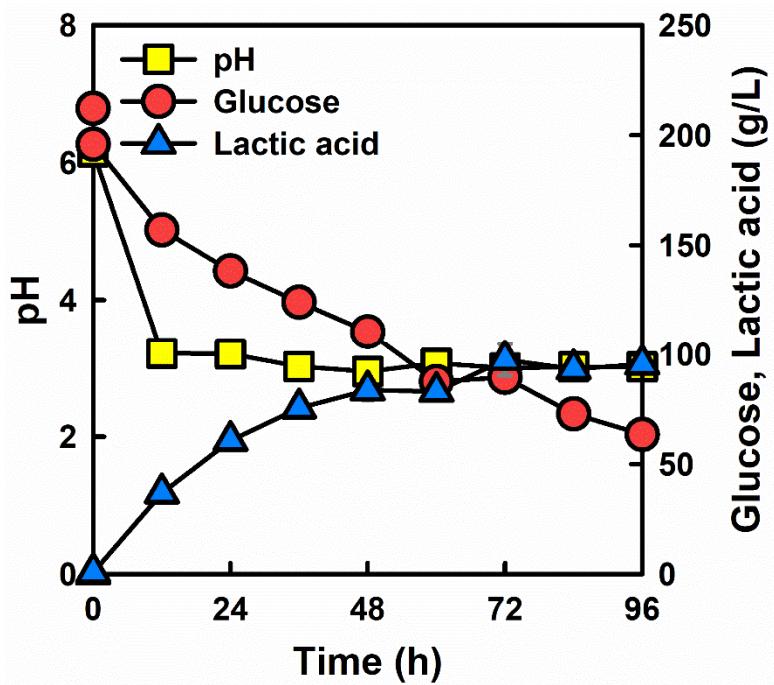


Figure S3. Fermentation profiles of the BK01 strain in complex medium containing 200 g/L glucose under oxygen limited conditions (80 rpm) with initial cell concentration of 10 g DCW/L (g dry cell weight/L).

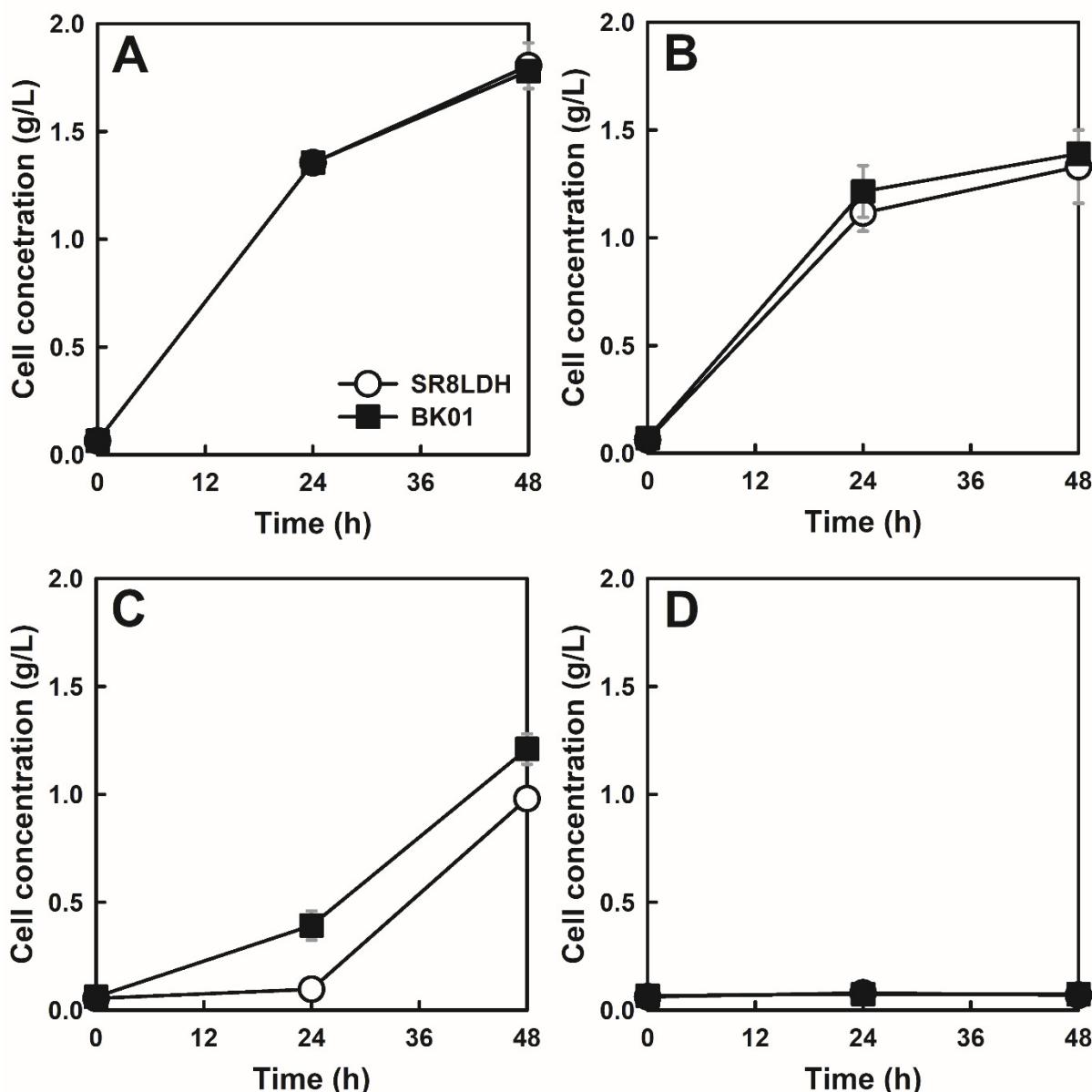


Figure S4. Comparison of volumetric growth rates (g/L-h) of the SR8 LDH and BK01 strains in complex medium containing 20 g/L of glucose and acetic acid. (A) 2 g/L, (B) 3 g/L, (C) 4 g/L, and (D) 5 g/L acetic acid is contained in complex medium. All experiments performed under oxygen limited conditions with cell concentration of 0.05 g DCW/L (g dry cell weight/L).

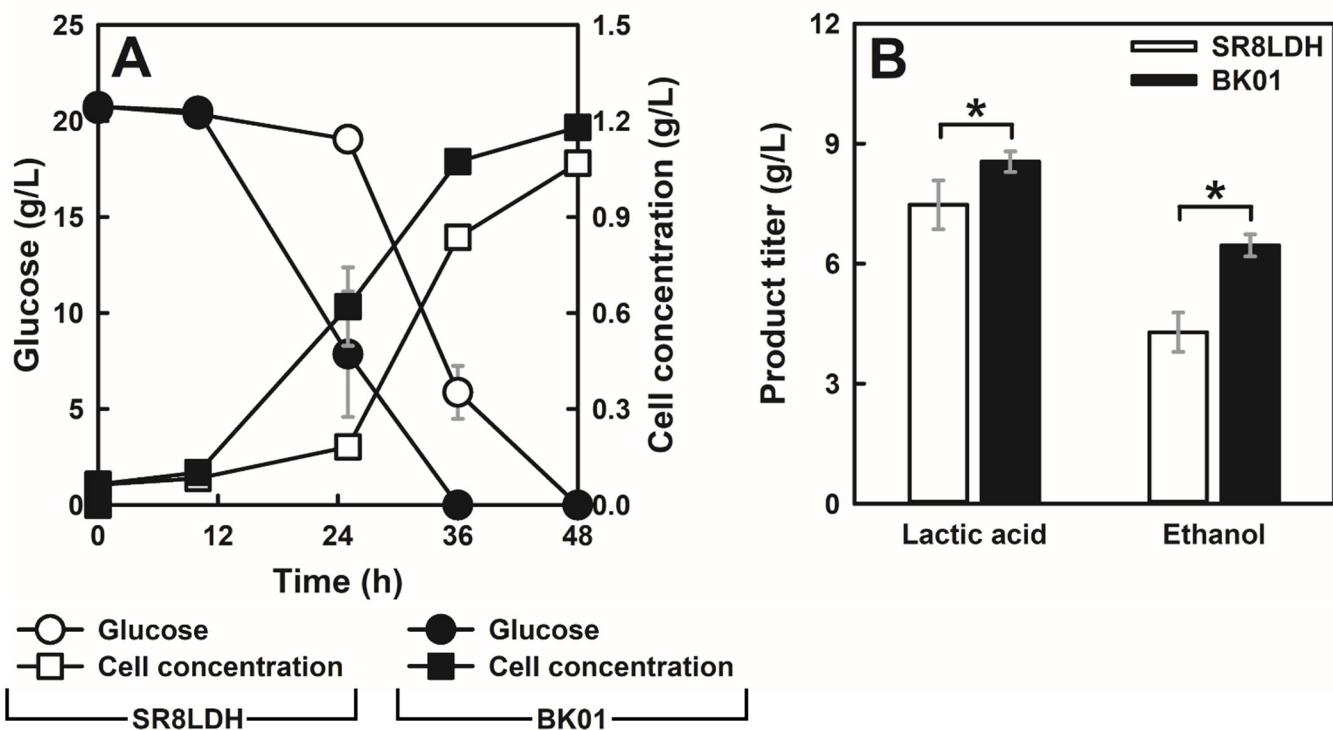


Figure S5. Fermentation profiles of the SR8LDH and BK01 strains in complex medium containing 20 g/L glucose and 4 g/L acetic acid. (A) Glucose consumption and cell concentration, (B) maximum product titers at 36 h. The values are the mean of three independent experiments, and the error bars indicate the standard deviations. All experiments were conducted under oxygen limited conditions (80 rpm), with an initial cell concentration of 0.05 g DCW/L. Asterisks denote statistically significant differences (Student's t-test, $p < 0.05$).

Table S1. Unique, non-synonymous SNPs in the parental and evolved strain

No	Chr	ORF	Gene	SNP detection by Illumina	Sanger confirmation
1	I	YAL063C	FLO9	1825 A>T	-
2	II	YBR112C	CYC8	1611 T>A	-
3	III	YCR018C	SRD1	289 G>A	PCR failure
4	IV	YDL248W	COS7	334 G>A 350_351 CT>TG 355 C>A 427 A>T 541 C>T 820 C>T 884 T>C	- - - - - - -
5	IV	YDL065C	PEX19	1024 C>A	-
6	IV	YDR150W	NUM1	2683 T>C	Tandom repeats
7	IV	YDR544C	-	161 G>C 167 G>C 173 G>C 179 G>C	Sequencing error Sequencing error Sequencing error Sequencing error
8	IX	YIL169C	CSS1	294_295 CT>AG 565 G>A 597_598 AG>TA 1347_1348 AC>GT 1363_1364 AC>GA	PCR failure PCR failure PCR failure PCR failure PCR failure
9	IX	YIR019C	FLO11	1699 A>C 2090 G>C 2180 G>C	Tandom repeats Tandom repeats Tandom repeats
10	VII	YGR023W	MTL1	722 T>C	-
11	VII	YGR295C	COS6	222 G>A 253 G>A 259 G>C 265 G>T	- - - -
12	VIII	YHR030C	SLT2	1142_1143 insertion ACA 1145_1146 insertion ACA	- -
13	VIII	YHR056C	RSC30	2014 A>G	-
14	VIII	YHR143W	DSE2	605_613 deletion CTTCTTCTT	-
15	VIII	YHR219W	-	455 T>C	-
16	X	YJR151C	DAN4	2746 A>G	-
17	XI	YKL164C	PIR1	297 A>T 302_303 AA>CT 305 C>T 310 G>C	- - - -
18	XII	YLL066C	-	2090 T>C 2054 T>C	- -
19	XII	YLL021W	SPA2	2760_2761 GA>AG 2767 G>A 2777 T>C	- - -
20	XII	YLR286C	CTS1	1430_1444 deletion GTAGTACAAGCTCAG	-
21	XII	YLR410W-B	-	2213 C>T	-
22	XIII	YML001W	YPT7	553 G>A	Confirmed
23	XV	YOL159C-A	-	172 C>A	Confirmed
24	XV	YOL155C	HPF1	493 A>T 506 A>C	- -

Table S2. Guide RNA structure

Description	Sequence
SNR52 promoter	TCTTGAAAAGATAATGTATGATTATGCTTCACTCATATTATACAGAAACTT-GATGTTTCTTCGAGTATATAACAAGGTGATTACATGTACGTTGAAGTACAACCTA-GATTGTAGTGCCTCTGGCTAGCGTAAAGGTGCG-CATTTCACACCCCTACAATGTTCTGTTCAAAAGATTGGTCAAACGCTGTAGAA-GTGAAAGTTGGTGCATGTTCGCGTTGAAACTCTCCGAGTGAAAGA-TAAATGATC
Target sequences	
YPT7.2	ATCATTATACTCATCTTCAA
YOL159C-A.3	ACTGTGAACTCGATTATCA
$\Delta yol159c-a.1$	GTACACCTACCCGTACCCGG
Structural crRNA	GTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT-GAAAAAGTGGCACCGAGTCGGTGGTGC
SUP4 terminator	TTTTTTGTTTTATGTCT

Table S3. Primers

Name	Description	Sequence
For donor DNA preparation for Cas9 engineering		
Kim637	mYPT7_F	AACGCCATAAACGTTGATACCGCATTGAAGAAATTGCAAGGAG-TGCTTTACAACAGAATCAAGCTGATA CAAAAGCTTTGAAGATGAC
Kim638	mYPT7_R	GAAGATACAATTAAGTAGTACAGCTAACAGCTACAA-GAATTATTTCTCCATCTAGGCGAATATTGATG
Kim679	mYOL159C-A_F	CTGCTTAAAGCGTAGAGTTC
Kim680	mYOL159C-A_R	CTCCAGTCGAGATAACGATAC
Kim770	Δ ypt7_F	GAATAAGCTCATCCAGTCCAC-TTCTTATCCATATAGAAACCCCTCTG-TATCAATTCAAATTAAGTGACT GTGAACTCGATTATCAGGG
Kim771	Δ ypt7_R	AAATTATTAATTTTTAAGGATACGCTATAAAGGATTACATAA-TAGAAGATACAATTAAGTAGTACAGC CCCTGATAAAATCGAGTTCAC
Kim684	Δ yol159c-a_F	ATAAGTACTATATACGCTGCTAGTTCTTGTGAGTTGG-GACCAAACCAATACCATTGTG GAATCACTGCACATGTACAC
Kim684	Δ yol159c-a_R	GTAAGTTACTGAGTCGACAACAGAGCTAGCAT-ACGCATGAATCTCGGTCTGAcctCCGGTGACGGGTAG GTG-TACATGTGCAGTGATT
For sequencing confirmation of Cas9 engineering		
Kim569	YPT7_F	AAGCAGTCGTTACAGAGGAACAG
Kim570	YPT7_R	AAAGTTCGGAACGCATACAC
Kim679	YOL159C-A_F	CTGCTTAAAGCGTAGAGTTC
Kim680	YOL159C-A_R	CTCCAGTCGAGATAACGATAC

Table S4. List of identified intracellular metabolites in SR8LDH and BK01

No.	Metabolite name	Retention time (min)	Average (Peak height)		Standard deviation (Peak height)		P-value	Fold-change
			SR8LDH	BK01	SR8LDH	BK01		
1	Galactonic acid	11.52	3159.83	5113.31	375.99	730.28	0.00	1.62
2	Inositol-4-monophosphate	14.10	21732.80	34975.02	5859.23	6685.40	0.00	1.61
3	Glucose-6-phosphate	13.51	17166.41	25448.42	3408.25	6555.88	0.02	1.48
4	Glutathione	12.69	18613.83	25816.39	1597.19	4945.13	0.01	1.39
5	Maltose	15.78	722539.37	934142.55	58749.37	91535.86	0.00	1.29
6	Galactose	10.81	887478.30	1126514.21	101369.02	147460.97	0.01	1.27
7	Glucose	10.97	650081.18	821610.70	84817.83	105729.50	0.01	1.26
8	Hydroxylamine	4.18	1707.76	2111.38	280.12	307.94	0.04	1.24
9	Leucine	5.76	802136.40	962540.06	44678.38	94589.39	0.00	1.20
10	Ethanolamine	5.75	799349.92	958225.85	44225.52	95076.02	0.00	1.20
11	Methylphosphate	4.83	2633.35	1952.75	307.12	185.23	0.00	-1.35
12	3-Phosphoglycerate	10.18	13890.24	10086.34	1407.66	2596.32	0.01	-1.38
13	Uridine	14.36	42067.14	30308.65	6697.43	4846.25	0.01	-1.39
14	Palmitoleic acid	11.78	44625.57	31809.04	7283.24	1735.48	0.00	-1.40
15	Malate	7.68	13091.78	9197.55	1618.43	2673.80	0.01	-1.42
16	Glycine	6.08	486113.47	336038.90	89401.35	26766.88	0.00	-1.45
17	Fructose-6-phosphate	13.41	10802.92	7164.25	1659.13	959.01	0.00	-1.51
18	UDP-N-acetylglucosamine	10.40	3235.75	2075.16	384.04	280.93	0.00	-1.56
19	Threitol	7.79	3490.23	2216.67	234.93	224.43	0.00	-1.57
20	Threonine	6.81	63817.49	32185.79	6042.87	4621.75	0.00	-1.98
21	Citric acid	10.26	44861.83	15176.83	9059.16	6098.41	0.00	-2.96
22	Galactinol	16.68	33903.50	10454.76	4446.48	1290.92	0.00	-3.24
23	Pyrophosphate	9.12	27142.39	4158.59	16507.64	2697.25	0.01	-6.53
24	N-acetylglutamate	10.08	6233.54	848.82	1508.54	226.20	0.00	-7.34

* Metabolites sorted by fold-change value

* The colored metabolites indicate the five metabolites with the highest (red) and the lowest (blue) fold-change value among significantly different metabolites between SR8LDH and BK01.