Engineering a Hypoxia-Tolerant *Saccharomyces cerevisiae* for Rapid Ethanol Production via Co-Utilization of Glucose and Acetic Acid and Redox-Enhanced Flocculation

Sadat Mohamed Rezk Khattab,*[abc§] Mohammed Oksh Mohammed,[ad§] Takashi Nagata,[ade] Takashi Watanabe[bf] and Masato Katahira*[adef]

Supplementary information

7 Pages

Five figures (Figs. S1~S5)

Four tables (Table S1, S2, S3, and S4)

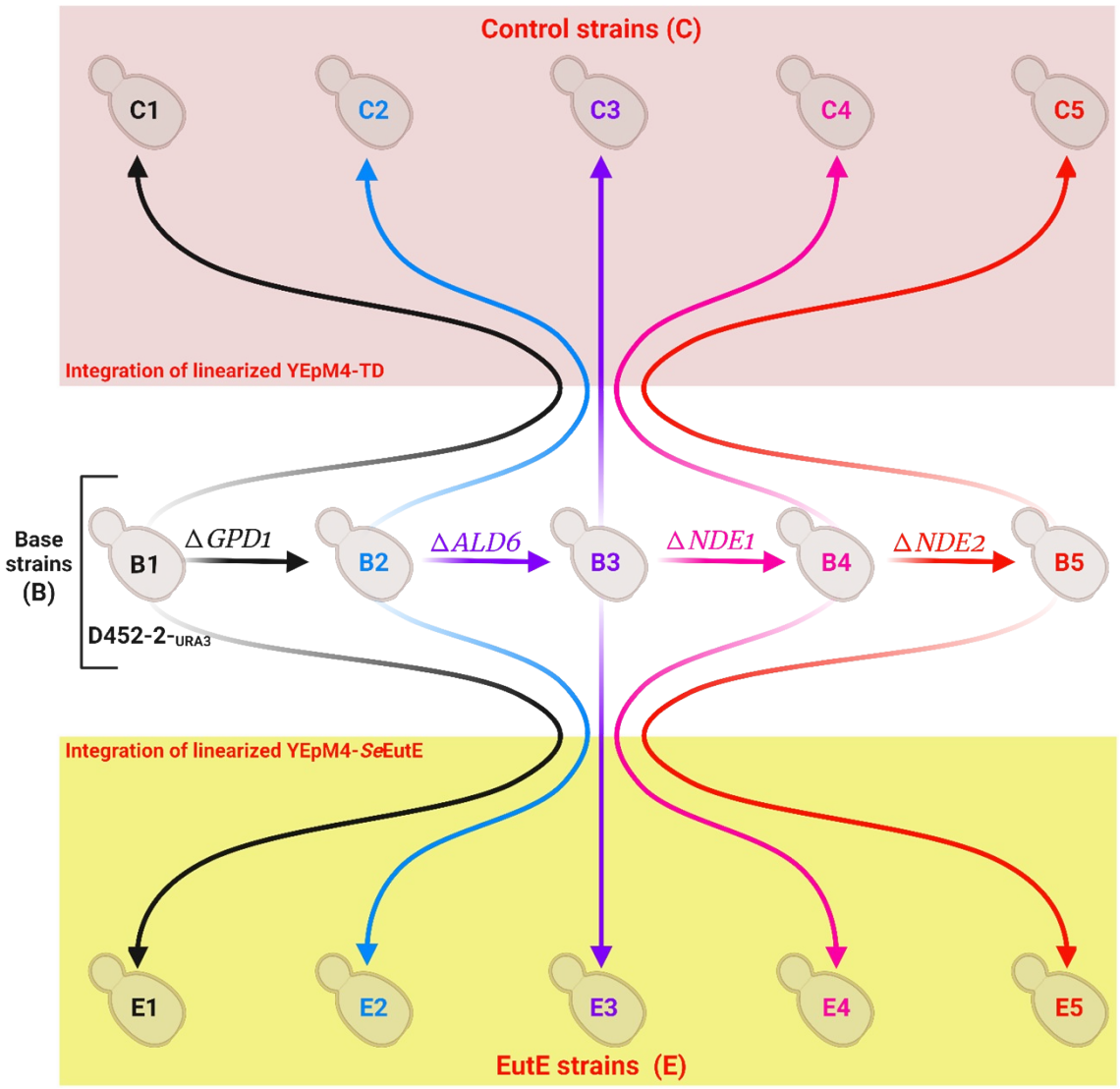

Fig. S1 Flow chart showing the progression of genetic modifications made and the names of the engineered strains used in this study. First, *S. cerevisiae* strain D452-2-*URA3* (B1) underwent sequential deletion of *GPD1*, *ALD6*, *NDE1*, and *NDE2* genes using CRISPR genome editing techniques, resulting in B2-B5. A linearized *Salmonella enterica* (*SeEutE*) cassette/*LEU2* plasmid was integrated into these base strains, B1-B5, to generate EutE strains E1-E5, respectively. The control strains C1-C5 were constructed by integrating an empty linearized cassette/*LEU2* plasmid. *GPD1* refers to the NAD-dependent glycerol-3-phosphate dehydrogenase 1 gene, *ALD6* represents the $NADP^+$-dependent cytosolic aldehyde dehydrogenase gene, and *NDE1* and *NDE2* denote mitochondrial external NADH dehydrogenase genes.

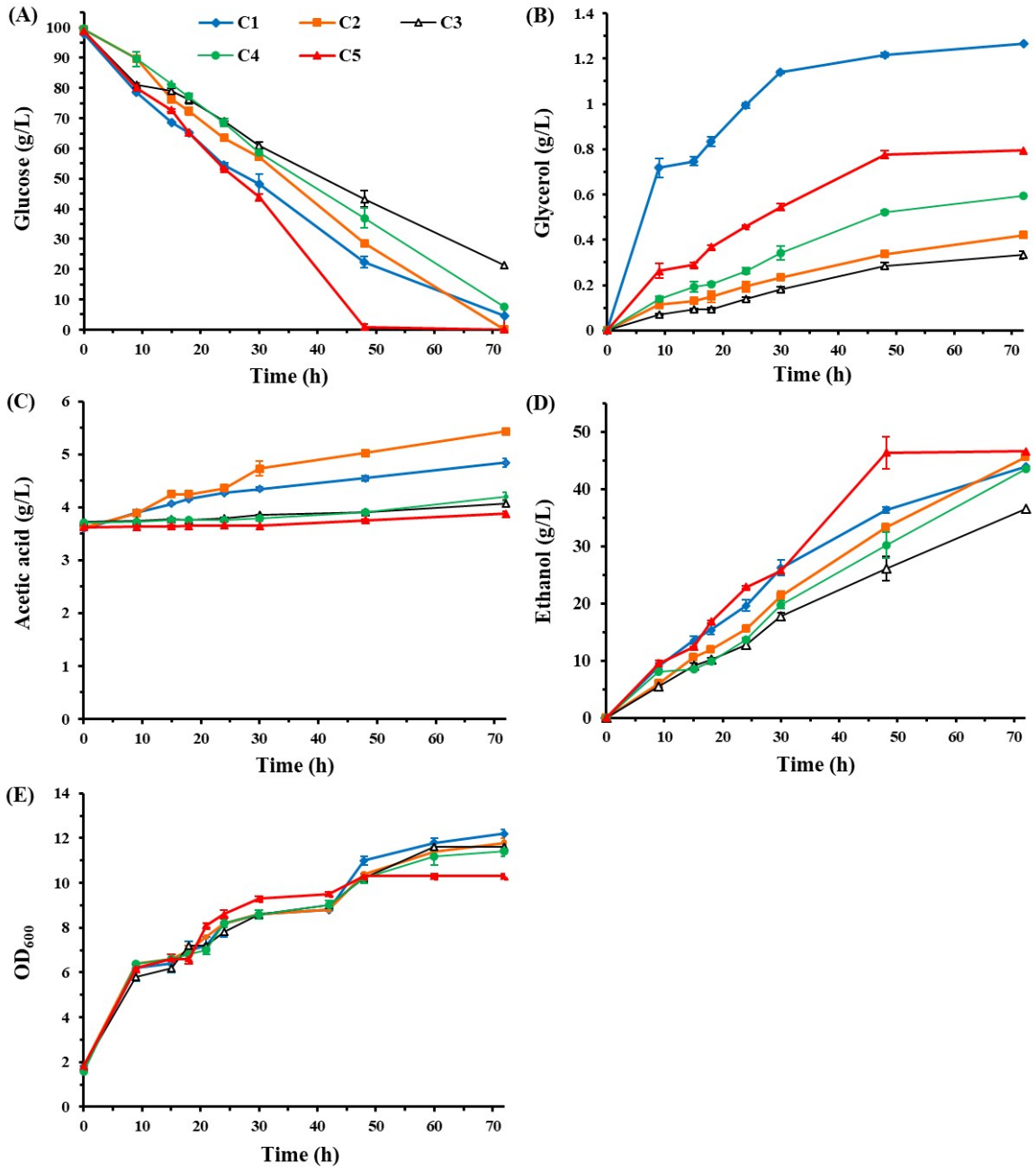

Fig. S2. Time course fermentation profiles of control strains C1–C5 in $YNB_{D100\text{-}AC4}$ medium (pH 5) under hypoxic conditions (V_c/V_f ratio of 50/100). Panels show: (A) glucose consumption, (B) glycerol production, (C) acetic acid concentration, (D) ethanol production, and (E) cell density (OD600). Error bars indicate standard deviation (n = 3). OD_{600} values for strain C5 were measured after de-flocculation using buffer (50 mM sodium acetate and 0.1 M EDTA) according to Kobayashi et al. (1998).

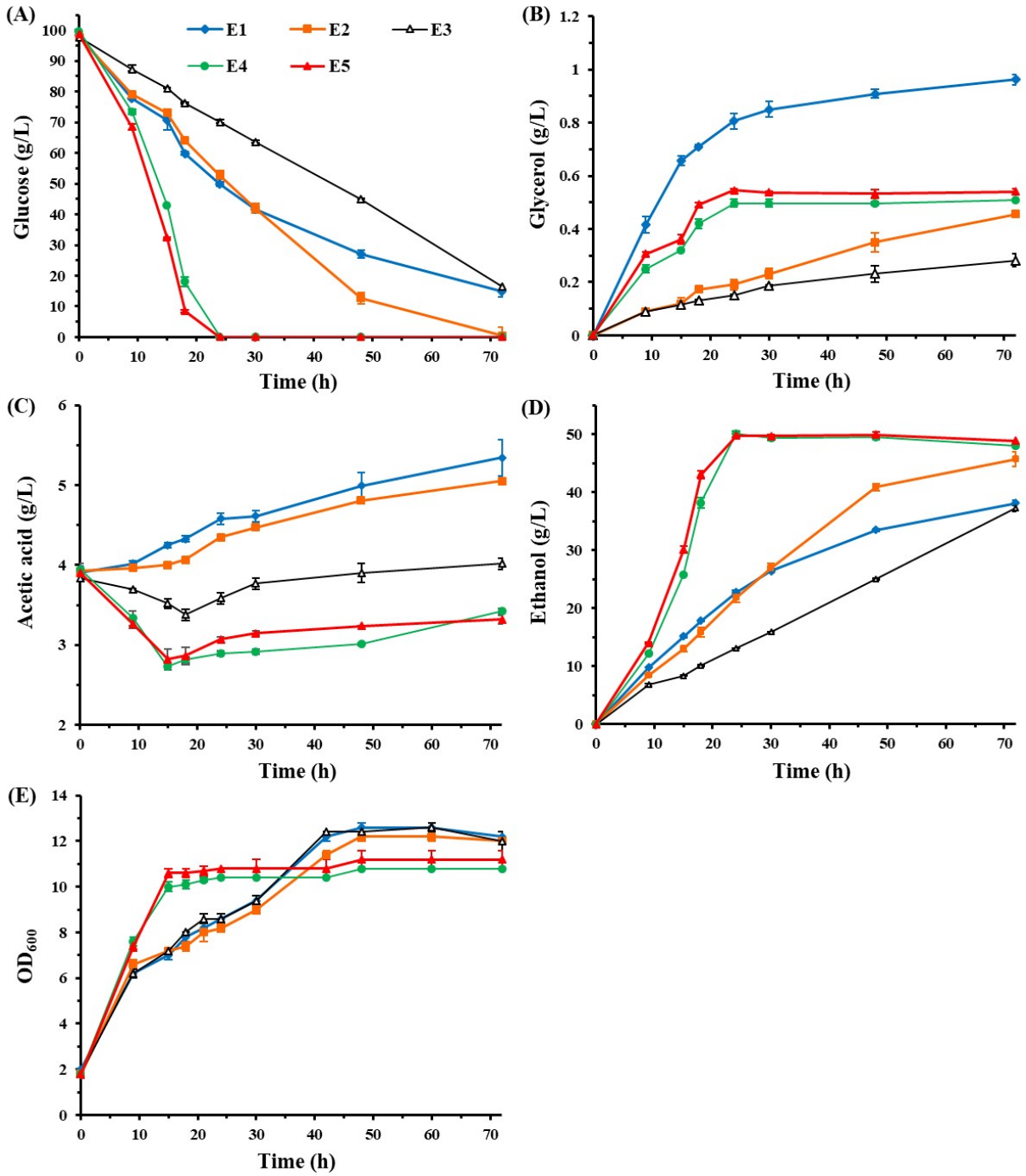

Fig. S3. Time course fermentation profiles of engineered EutE strains E1–E5 in $YNB_{D100\text{-}AC4}$ medium (pH 5) under hypoxic conditions (V_c/V_f = 50/100). Panels show: (A) glucose consumption, (B) glycerol production, (C) acetate concentration, (D) ethanol production, and (E) cell density (OD_{600}). Error bars indicate standard deviation (n = 3). OD_{600} values for strains E4 and E5 were measured after de-flocculation using buffer (50 mM sodium acetate and 0.1 M EDTA) as described by Kobayashi et al. (1998).

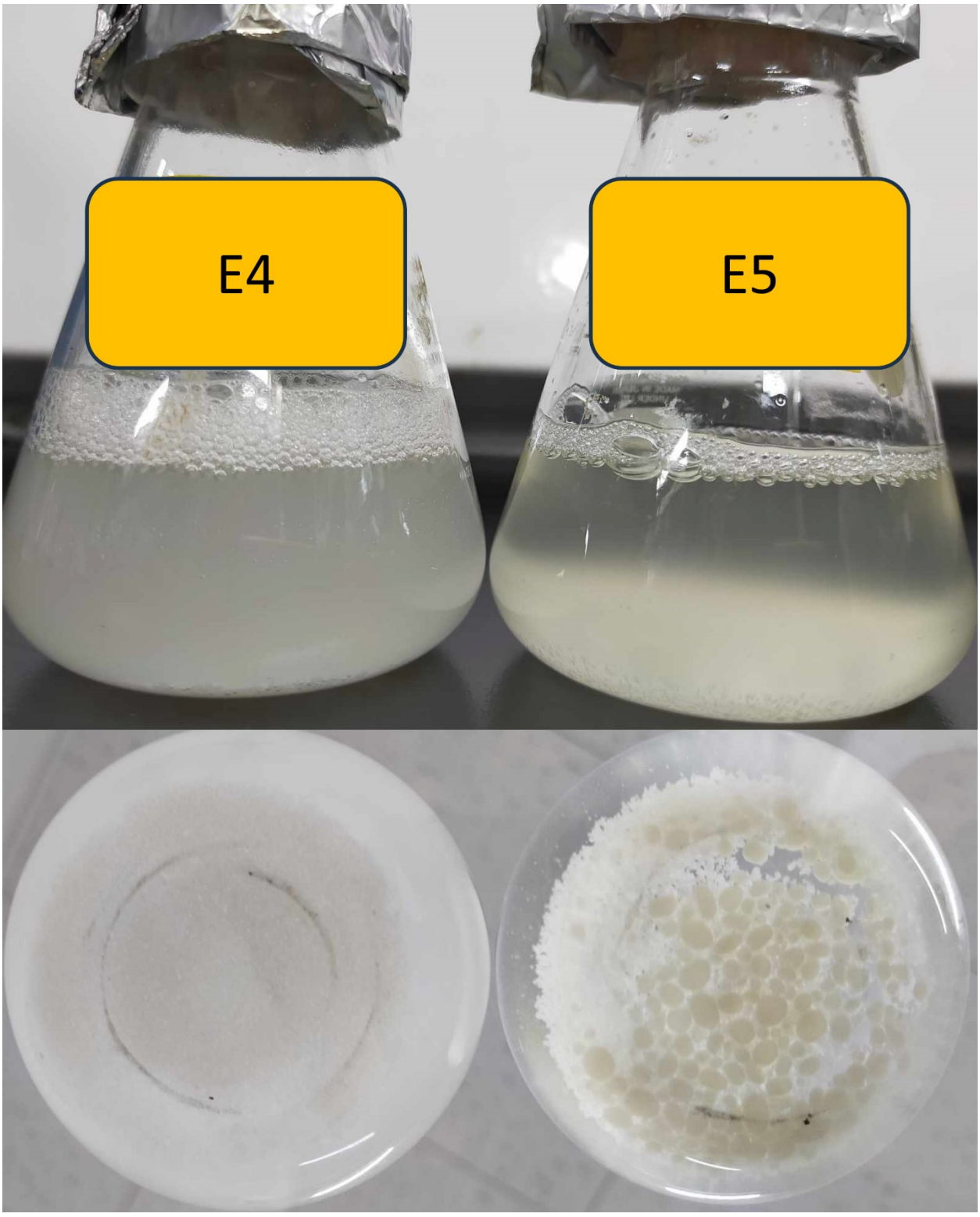

Fig. S4 Comparative observation of flocculation behavior and floc size between engineered strains E4 and E5.

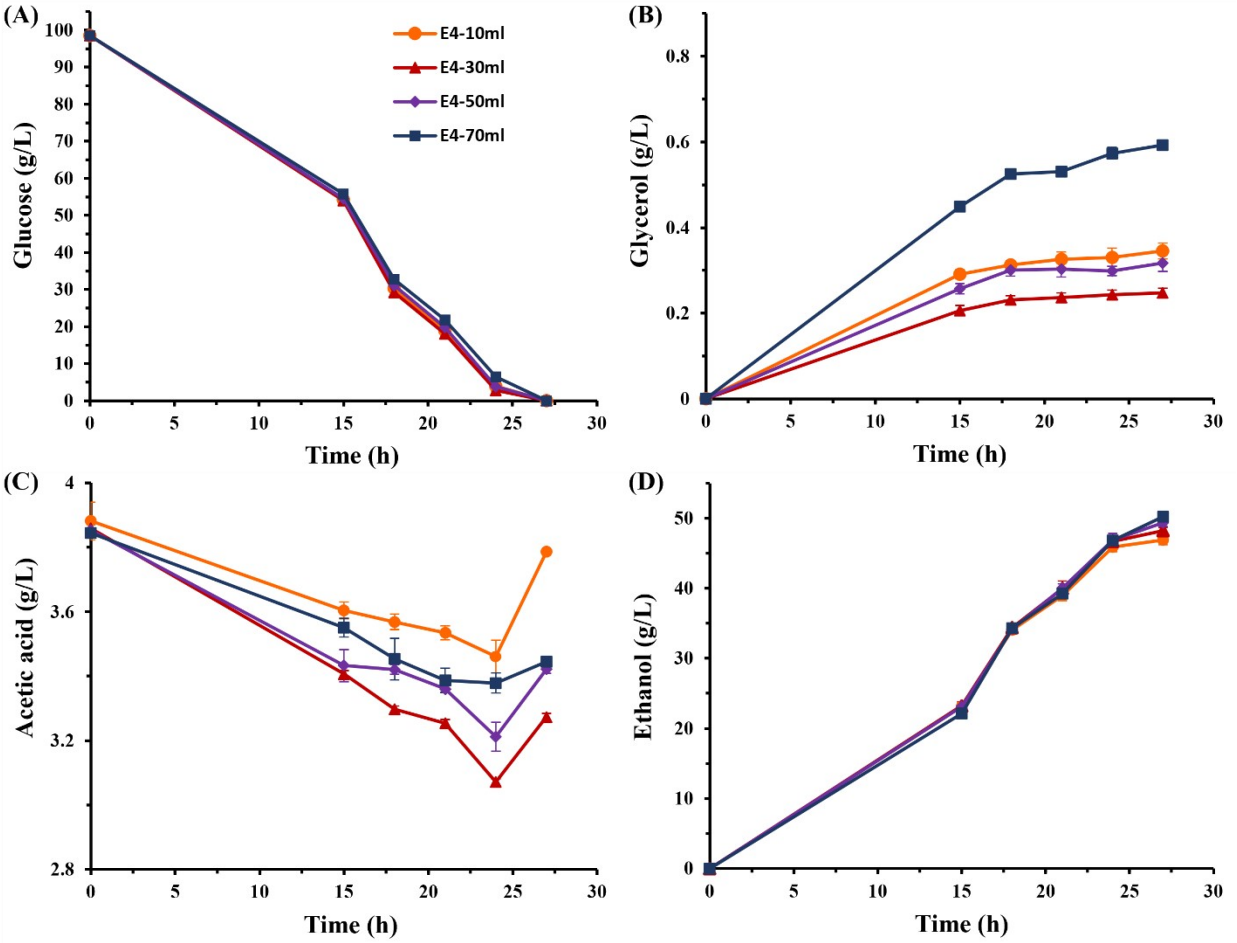

Fig. S5 Time course fermentation of strain E4 in $YNB_{D100\text{-}AC4}$ medium (pH 5) under varying oxygenation conditions: hyperoxic (V_c/V_f = 10/100) and hypoxic (V_c/V_f = 30/100 and 70/100). Panels show: (A) glucose consumption, (B) glycerol production, (C) acetic acid concentration, and (D) ethanol production. Error bars indicate standard deviation (n = 3).

Table S1. Yeast nitrogen base components used in this study.

Component	Quantity per liter
Ammonium sulfate	5 g
Biotin	0.002 mg
Calcium pantothenate	0.4 mg
Folic acid	0.002 mg
Inositol	2 mg
Niacin	0.4 mg
Para-aminobenzoic acid	0.2 mg
Pyridoxine HCl	0.4 mg
Riboflavin	0.2 mg
Thiamine HCl	0.4 mg
Boric acid	0.5 mg
Copper sulfate	0.04 mg
Potassium iodide	0.1 mg
Ferric chloride.6H2O	0.2 mg
Manganese sulfate.H2O	0.4 mg
Sodium molybdate.2H2O	0.2 mg
Zinc sulfate.H2O	0.4 mg
Potassium phosphate monobasic	1 g
Magnesium sulfate	0.5 g
Sodium chloride	0.1 g
Calcium chloride	0.1 g

Table S2. Primers used in this study.

Primers for constructing cassettes and plasmids	
Sal1-TDH3p FOR	Aaa**gtcgac**ttttcagttcgagtttatca
Not1-TDH3p REV	Tcat**gcggccgc**tttgtttgtttatgtgtg
NotI-SeEutE FOR	Aca**gcggccgc**atgaaccaacaggacatag
BamHI-SeEutE REV	Ggg**ggatcc**ttagacaatacggaaagcatc
Not1-BamHI-DIT1t$_{d22}$ FOR	Ttttt**gcggccgcggatcc**taaagtaagag
Xho1-DIT1t$_{d22}$ REV	Aaaa**ctcgag**gatgaaaagaaaggcaaat
SeEutE Seq1 FOR	Caagtgtcacaccatctactaaccc
SeEutE Seq1 FOR	Tgataacaatataatatgcgc
Primers for constructing multiplex pCas9 plasmids	
pCas9-gRNA target 1120-*ALD6* FOR	Ggcgccaagatcttaactgggttttagagctagaaatagc
pCas9-gRNA target 1120-*ALD6* REV	Ccagttaagatcttggcgccaaagtcccattcgccacccg
pCas9-gRNA target 59-*NDE1* FOR	gctctgtaggctaaacctgggttttagagctagaaatagc
pCas9-gRNA target 59-*NDE1* REV	Ccaggtttagcctacagagcaaagtcccattcgccacccg
pCas9-gRNA target 369-*NDE2* FOR	Gtctttgtataacgtgaccggttttagagctagaaatagc
pCas9-gRNA target 369-*NDE2* REV	Cggtcacgttatacaaagacaaagtcccattcgccacccg
pCas9 FOR	cggaataggaacttcaaagcg
pCas9 REV	ttttttctgcagcgaggagcc
Upstream *ScGPD1-TDH3*p FOR.	aa**ctcgag**tgtatattgtacacccccccctccacaaacacaaatattgataatataaag**cccgggagctc**agttcgagt
downstream *ScGPD1-DIT1t*$_{d22}$ REV	tt**gtcgac**gaaaaaagtgggggaaagtatgatatgttatctttctccaataaat**ccgcgg**gatgaaaagaaaggcaaat a
Upstream *ScALD6-TDH3*p FOR	aaaactcgagaaaaaacatcaagaaacatctttaacatacacaaacacatactatcagaatttttcagttcgagtttatc
Downstream *ScALD6-DIT1t*$_{d22}$ REV	tttgtcgaccaagtaagtttatatgaaagtattttgtgtatatgacggaaagaaatgcaggatgaaaagaaaggcaaata
Upstream *ScNDE1-TDH3*p FOR	ttcttcatatagcatcataatattgacttattagagcaaataaaaaattaaccaataatatcagttcgagtttatcatta
Downstream *ScNDE1-DIT1t*$_{d22}$ REV	taaaataaaaaacaaaactgatcatcatttaaaaatgttattctcttgtatctatttctagatgaaaagaaaggcaaata
Upstream *ScNDE2-TDH3*p FOR.	gattgtaccaggaacatagtagaaagacaaaaacaaccaccgtacttgccattcgtatagtcagttcgagtttatcatta
Downstream *ScNDE2-DIT1t*$_{d22}$ REV	tataatacgtaaatgatagtaaacaatttgaattggaacaatgaatatataaaacaaggagatgaaaagaaaggcaaa ta
Primers used for colony PCR to confirm the homologous integration after knock-out	
282-downstream *ScALD6*	ggatatagcagttgttgtacactagc
330-upstream *ScNDE1*	cttttgtgctgttggggctagaactataag
127-upstream *ScNDE2*	ggagtgcgctgttgacctgcgtatataag

Table S3. Names, relevant genotypes, and references of plasmids used in this study.

Plasmids	Relevant genotype	Reference
YEpM4	*LUE2*	15
YEpM4-TD	*LUE2, TDH3* promoter and mutated $DIT1_{d22}$ terminator	This study
YEpM4-*Se*EutE	*LUE2,* expresses *SeEutE*	This study
Multiplex pCas9/GPD1-1	Multiplex expresses Cas9 and gRNA to base No. 135 of *GPD1*	16
Multiplex pCas9/GPD1-2	Multiplex expresses Cas9 and gRNA to base No. 1045 of *GPD1*	16
Multiplex pCas9/ALD6	Multiplex expresses Cas9 and gRNA to base No. 1120 of *ALD6*	This study
Multiplex pCas9/NDE1	Multiplex expresses Cas9 and gRNA to base No. 59 of *NDE1*	This study
Multiplex pCas9/NDE2	Multiplex expresses Cas9 and gRNA to base No. 369 of *NDE2*	This study

Table S4 The sequence of the *TDH3p*-*SeEutE*-*DIT1t*$_{d22}$ cassette.

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