Supplementary information

High-titer bioethanol production from steam exploded corn stover by an engineering *Saccharomyces cerevisiae* strain with high inhibitors tolerance

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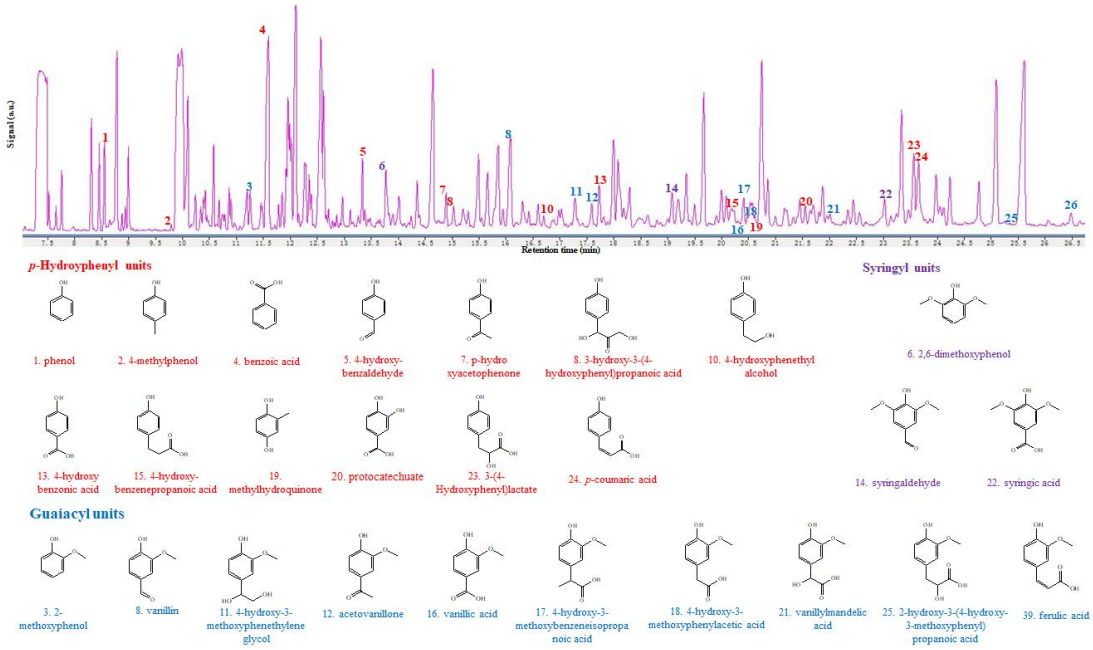

Figure S1. Lignin monomers presented in SECSH detected by GC/MS.

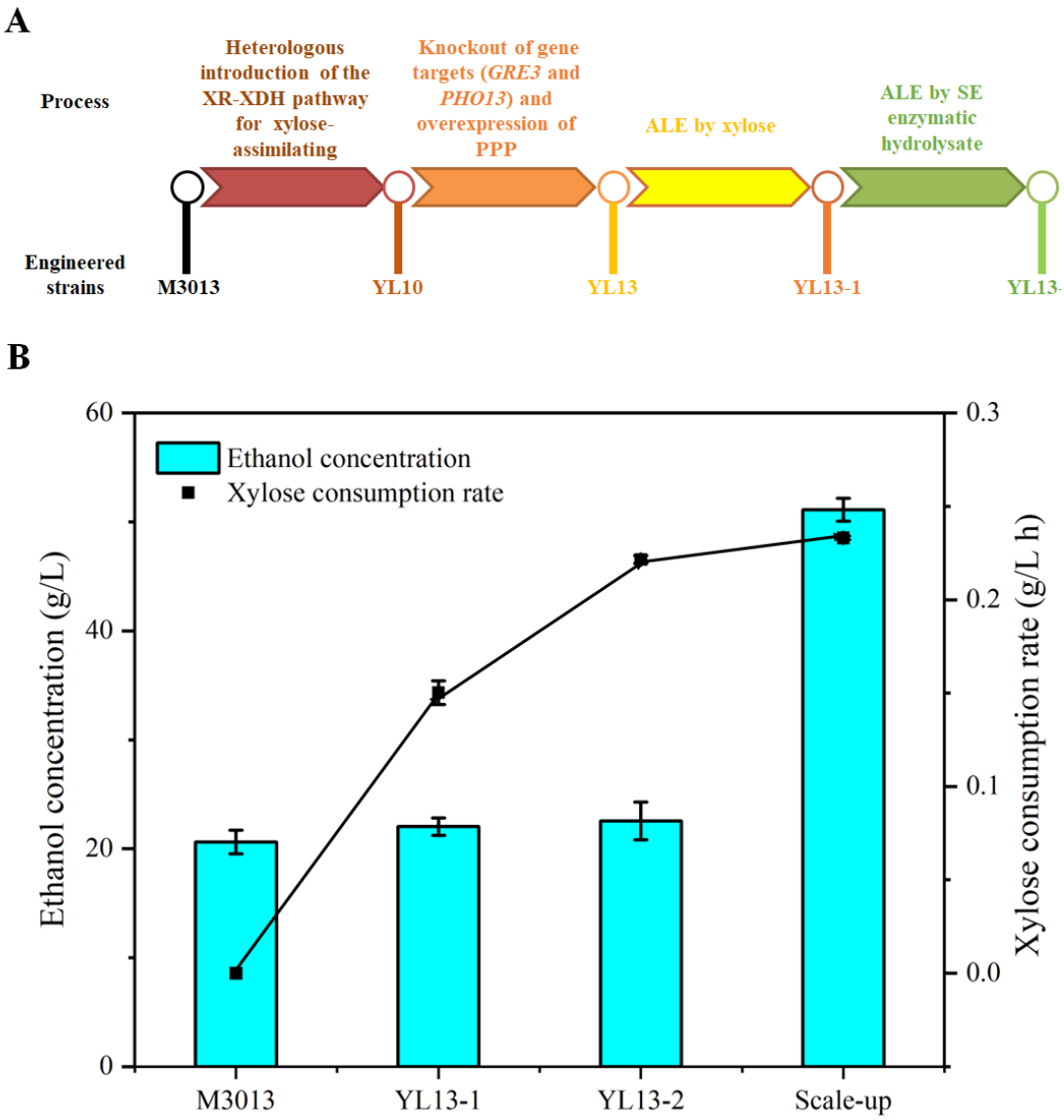

Figure S2. Construction and the evaluation of the genetic strain. (A) Flowchart of the construction process of *Saccharomyces cerevisiae* YL13-2. (B) Xylose consumption rate and ethanol concentration after batch fermentation of YL13-2 strain using the synthetic medium.

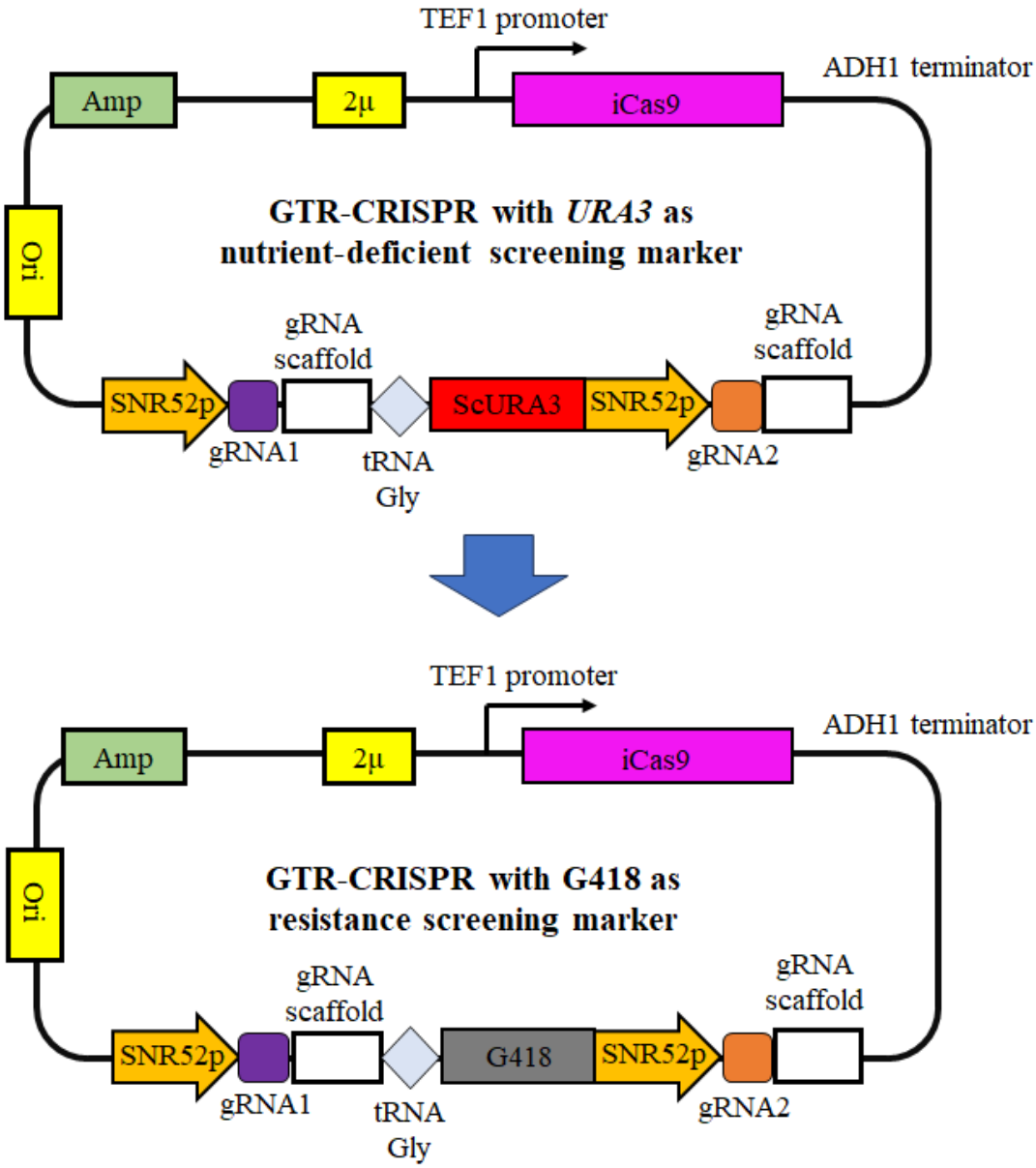

Figure S3. A gRNA-tRNA array for CRISPR-Cas9 (GTR-CRISPR). The screening marker replaced by *URA3* for *G418*.

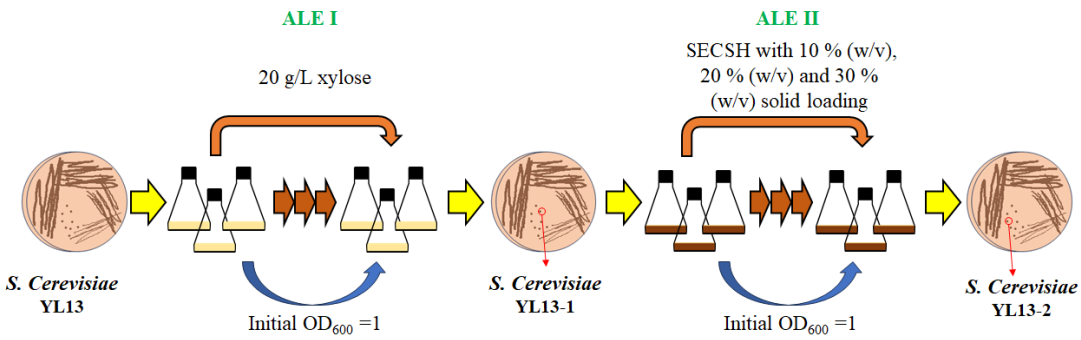

Figure S4. Schematic diagram of the xylose-assimilating enhancement and the inhibitors tolerance increases of *S. cerevisiae* YL 13 by the ALE strategy.

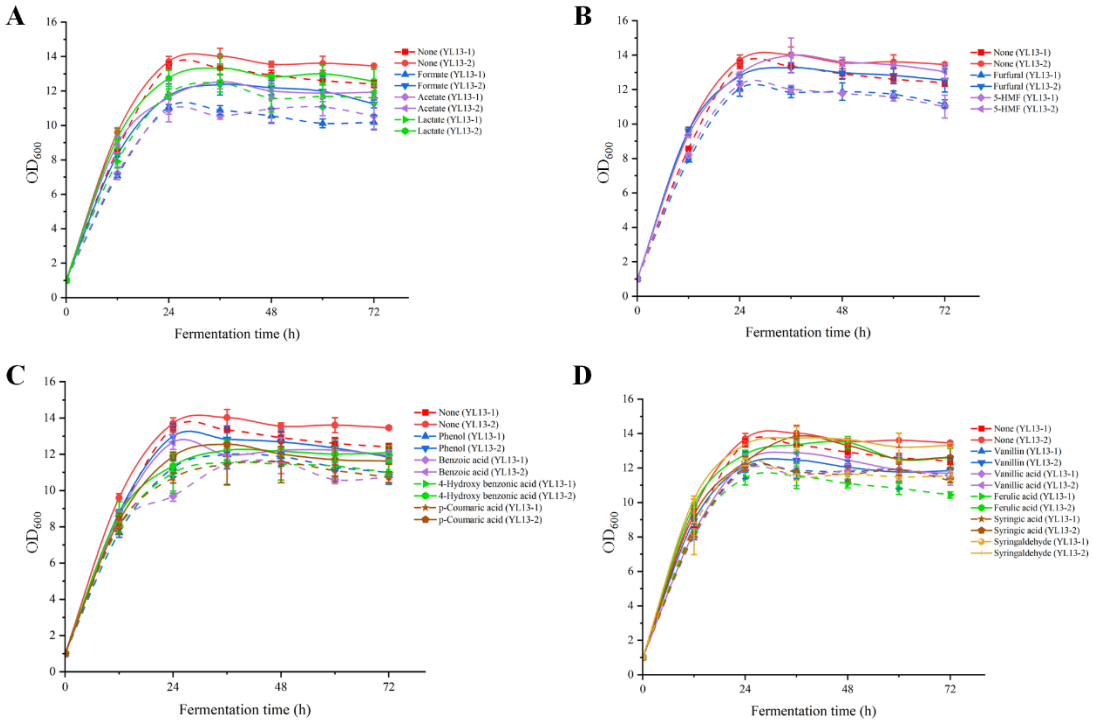

Figure S5. (A) Growth curves of *S. cerevisiae* YL13-1 and YL13-2 in synthetic medium containing organic acids that presence in SE enzymatic hydrolysate. (B) Growth curves of *S. cerevisiae* YL13-1 and YL13-2 in synthetic medium that containing furan derivates (0.5 g/L of furfural and 5-HMF) presence in SE enzymatic hydrolysate. (C) (D) Growth curves of *S. cerevisiae* YL13-1 and YL13-2 in synthetic medium containing phenolic compounds (0.1 g/L of phenol, benzoic acid and 4-(hydroxymethyl)benzaldehyde, 0.04 g/L of *p*-coumaric acid, vanillin, vanillic acid, ferulic acid, syringic acid and syringaldehyde) presence in SE enzymatic hydrolysate.

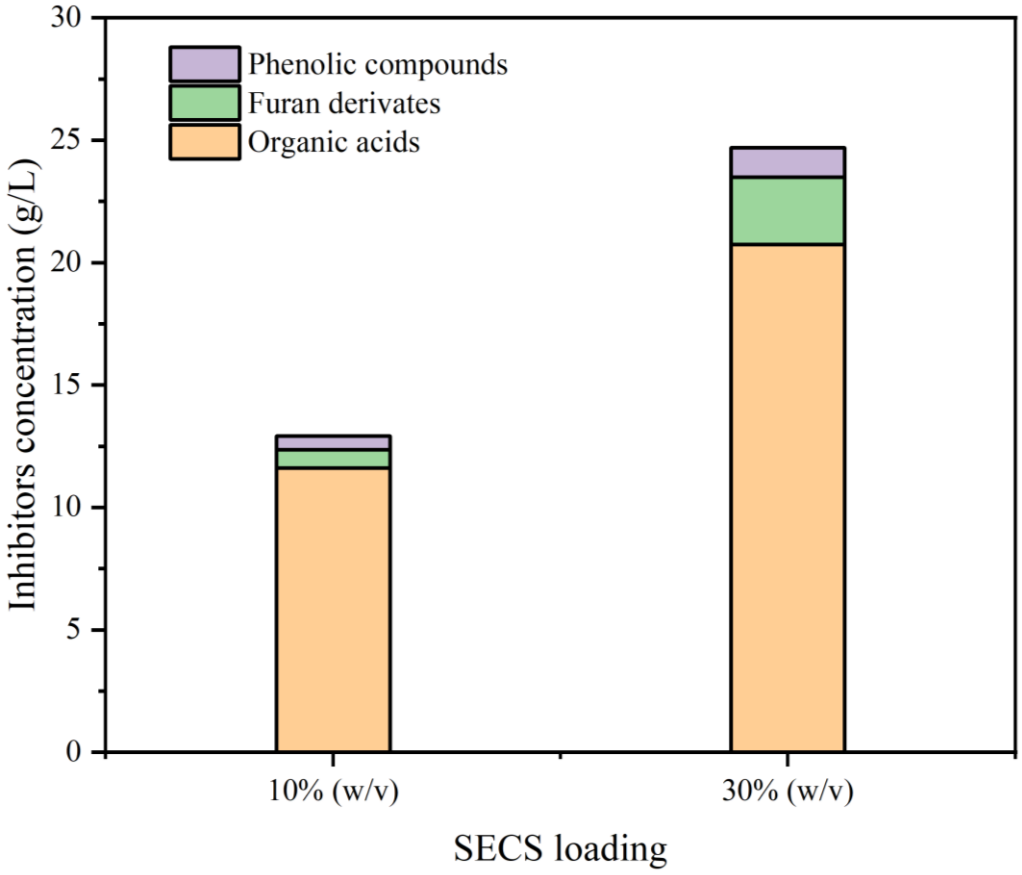

Figure S6. Inhibitors (organic acids, furan derivates and phenolic compounds) concentration in 10 % (w/v) and 30 % (w/v) of SECS loading.

Table S1. Strains involved in this study.

Strain	Relevant Genotype and Manipulation
M3013	Laboratory stored for bioethanol fermentation.
YL10	M3013 derivative, XI-3:: *TEF2p-**XYL1**-TPI1t-TEF1p-**mXYL1**-PGI1t-PGK1p-**XYL2**-CYC1t-HXT7p-**XKS1**-HXT7t*
YL11	YL10 derivative, ***PHO13***::gRNA
YL12	YL11 derivative, ***GRE3***::gRNA
YL13	YL12 derivative, XII-2:: *TDH1p-**RPE1**-PGI1t- PGK1p-**TAL1**-Hxt7t-TEF1p-**RKI1**-tCYC1-TDH3p-**TKL1**-GPDt*
YL13-1	Single-colony isolate from adaptive laboratory evolution in **xylose**; based on YL13
YL13-2	Single-colony isolate from adaptive laboratory evolution in **SE enzymatic hydrolysate**; based on YL13-1

Table S2. Primers involved in this study.

Primer No.	Name	Nucleotide sequence (5' >3')
P1	Gi-TDH1p-F	CTATAGGGCGAATTGGGTACCGAATAGGATATGCGACGAAGACGC
P2	Gi-TDH1p-R	GTTTGACCATATTTTGTTTTGTGTGTAAATTTAGTGAAGTACTG
P3	Gi-RPE1-F	CAAAACAAAATATGGTCAAACCAATTATAGCTCCCAG
P4	Gi-RPE1-R	CGACTCTAGACTAATCTAGCAAATCTCTAGAACGCAAT
P5	Gi-PGI1t-F	GCTAGATTAGTCTAGAGTCGACAACAAATCGCTCT
P6	Gi-PGI1t-R	GCTTATCGATACCGTCGACCTCGAGCATATGGGTATACTGGAGGCTTCAT
P7	RPE1-I-F	GCGCGTAACCACCACACC
P8	RPE1-I-R	ACTCATTAGGCACCCCAGGC
P9	Gi-PGK1p-F	CGAGGTCGACGGTATCGATAAGCTTGGAAGTACCTTCAAAGAATGGGGTC
P10	Gi-PGK1p-R	GTTCAGACATTTGTTTTATATTTGTTGTAAAAAGTAGATAATTACTTCCTTG
P11	Gi-TAL1-F	ATATAAAACAAATGTCTGAACCAGCTCAAAAGAAACA
P12	Gi-TAL1-R	CAAAGAATTCTTAAGCGGTAACTTTCTTTTCAATCAAGTC
P13	Gi-Hxt7t-F	TACCGCTTAAGAATTCTTTGCGAACACTTTTATTAATTCATG
P14	Gi-Hxt7t-R	TGGATCCCCCGGGCTGCAGGAATTCATAACTGACTCATTAGACACTTTTTGAAGC
P15	TAL1-I-F	TGTGCACTAATAGTTTAGCGTCG
P16	TAL1-I-R	ACTCATTAGGCACCCCAGGC
P17	Gi-pTEF1-F	CAGTTATGAATTCCTGCAGCCCGGGCACACACCATAGCTTCAAAATGTTTCT
P18	Gi-pTEF1-R	CGGCAGCCATCTTAGATTAGATTGCTATGCTTTCTTTCTAATGAG
P19	Gi-RKI1-F	CTAATCTAAGATGGCTGCCGGTGTCCCA
P20	Gi-RKI1-R	TAGAGCGGATTCACTTTTCGGTAACTTCAACACTACCGTC
P21	Gi-tCYC1-F	CGAAAAGTGAATCCGCTCTAACCGAAAAGGA
P22	Gi-tCYC1-R	CGGTGGCGGCCGCTCTAGAACTAGTCTTCGAGCGTCCCAAAACC
P23	RKI1-I-F	CAGAAAGTTGAGTGGGACGGAGA
P24	RKI1-I-R	ACTCATTAGGCACCCCAGGC
P25	Gi-TDH3p-F	TCGAAGACTAGTTCTAGAGCGGCCGCTTTGTTTGTTTATGTGTGTTTATTCGAAACTAAGT
P26	Gi-TDH3p-R	ATTGAGTCATAACAGTTTATTCCTGGCATCCACT
P27	Gi-TKL1-F	ATAAACTGTTATGACTCAATTCACTGACATTGATAAGCT
P28	Gi-TKL1-R	GTAAATTCACTTAGAAAGCTTTTTTCAAAGGAGAAATTAGCTTG
P29	Gi-GPDt-F	AGCTTTCTAAGTGAATTTACTTTAAATCTTGCATTTAAATAAATTTTCT
P30	Gi-GPDt-R	AGGGAACAAAAGCTGGAGCTCGGAATCTGTGTATATTACTGCATCTAGATATATGT
P31	TKL1-I-F	GGTAATTCTGACGGTAGTGTTGAAGTTAC
P32	TKL1-I-R	ACTCATTAGGCACCCCAGGC
P33	Donor-4xyxk-F	AGTAGAAATGCAGAGTGTGCTATATGTCCAATCTCGGTTTTGTAGTTTGGCATATGCTATATAACAGTTGAAATTTGGATA
P34	Donor-4xyxk-R	GATGGTTAGATGGCTGAATAAAAACGTTAATTAAAGGAATACAGAAGAGGACGCACAGATATATAACATCTGCATAATAGGCA
P35	Donor-RPE1-	GGATGTGATTGAACCATTTATTGATAATATCAAGGCTTTGA

	F	ATCAATCAAGAATAGGATATGCGACGAAGACGCTTCTGC
P36	Donor-TKL1-R	TTGTGCGCGTAAAAAACGGAACCAGAAGAAGCCAAAAAA AAAAGAAATTCGGAATCTGTGTATATTACTGCATCTAGATA TATGT
P37	Donor-GRE3-F	AAGACGATTGGGAAAATACTGTAATATAAATCGTAAAGGA AAATTGGAAATTTTTTAAAGTCCAGCCAGTAAAATCCATA
P38	Donor-GRE3-R	AGCATCGGAATGAGGGAAATTTGTTCATATCGTCGTTGAGT ATGGATTTTACTGGCTGGACTTTAAAAAATTTCCAATTT
P39	Donor-PHO13-F	AGCCAAATCACAAAAAAAGCCTTATAGCTTGCCCTGACAA AGAATATACAACTCGGGAAAAGGAGCAATGCAAAATCTAG
P40	Donor-PHO13-R	AAACCTGAATATTTTTCCTTTTCAAAAAGTAATTCTACCCC TAGATTTTGCATTGCTCCTTTTCCCGAGTTGTATATTCT
P41	Goden-XI-3-F	AAAGGTCTCTGATCCCCAACCGGCTGCTTTCATGGTTTTAG AGCTAGAAATAGCAAGTTAAAATAAGGCT
P42	Goden-XI-3-R	AAAGGTCTCTAAACCCACATGAAAGCAGCCGGTTGATCAT TTATCTTTCACTGCGGAGAAGT
P43	Goden-GRE3-F	AAAGGTCTCTGATCCCAGGATTCTATACGGGCGCGTTTTAG AGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC
P44	Goden-GRE3-R	AAAGGTCTCTAAACCGGGCTTGATTCTACAACCAGATCAT TTATCTTTCACTGCGGAGAAGTTT
P45	Goden-PHO13-F	AAAGGTCTCTGATCATGGGGTACGAATCTCTAGGGTTTTAG AGCTAGAAATAGCAAGTTAAAATAAGGCT
P46	Goden-PHO13-R	AAAGGTCTCTAAACGCAGTGTAACAGCCAAACGGGATCAT TTATCTTTCACTGCGGAGAAGT
P47	Cas9-I-F	CGATTTTTGTGATGCTCGTCAGGG
P48	Cas9-I-R	AAAAAGAAGAGAAAGGTCTGACTCGAG

Table S3. Plasmid and gRNA involved in this study.

Name	Relevant properties or genotype	Source
pRS425	ori, AmpR, 2μ, LEU2, lacZ	Laboratory preservation
pRS416	ori, AmpR, CEN/ARS, URA3, lacZ	Laboratory preservation
pRS01	pRS425 carrying *XYL1*, *mXYL1*, *XYL2*, *XKS1*	[1]
pRS02	pRS416 carrying *RPE1*, *TAL1*, *RKI1* and *TKL1*	This work
pLacZ-SalI(Cas9)	2μ,AmpR,TEF1p-*iCas9*-SNR52p	Laboratory preservation
pLacZ-XI-3	ori, AmpR, 2μ, G418, pTEF1-*iCas9*-tADH1, pSNR52-(XI-3 gRNA)-gRNA scaffold-tSNR52 **20bp Target Sequence**: CCCAACCGGCTGCTTTCATG, AACCGGCTGCTTTCATGTGG	This work
pLacZ-*GRE3*	ori, AmpR, 2μ, G418, pTEF1-*iCas9*-tADH1, pSNR52-(GRE3 gRNA)-gRNA scaffold-tSNR52 **20bp Target Sequence**: CCAGGATTCTATACGGGCGC, TGGTTGTAGAATCAAGCCCG	This work
pLacZ-*PHO3*	ori, AmpR, 2μ, G418, pTEF1-*iCas9*-tADH1, pSNR52-(PHO13 gRNA)-gRNA scaffold-tSNR52 **20bp Target Sequence**: ATGGGGTACGAATCTCTAGG, CCGTTTGGCTGTTACACTGC	This work

Table S4. Key parameters of batch ethanol fermentation by different strains using the synthetic medium containing inhibitors.

		Organic acids			Furan derivates			Phenolic compounds							
***S. cerevisiae* M3013**	**None**	**Formate**	**Acetate**	**Lactate**	**Furfural**	**5-HMF**	**Phenol**	**Benzoic acid**	**4-Hydroxy benzonic acid**	***p*-Coumaric acid**	**Vanillin**	**Vanillic acid**	**Ferulic acid**	**Syringic acid**	**Syringaldehyde**
Concentration (g/L)	/	5	8	5	0.5	0.5	0.1	0.1	0.1	0.04	0.04	0.04	0.04	0.04	0.04
OD_{600max}	13.31± 0.56	10.46± 0.16	9.16± 0.51	12.42± 0.25	11.72± 0.73	12.12± 0.47	11.81± 0.28	7.47± 0.33	10.77± 0.31	10.66± 0.28	10.83± 0.19	10.50± 0.37	11.10± 0.34	10.93± 0.15	10.68± 0.16
Ethanol concentration (g/L)	21.47± 0.84	20.38± 0.18	19.25± 0.68	20.98± 0.77	20.43± 0.42	19.69± 1.02	19.83± 0.66	19.01± 0.38	17.70± 0.60	16.02± 0.50	18.42± 0.05	16.52± 0.10	15.44± 0.21	16.08± 1.20	17.01± 0.43
Ethanol yield (g/g)	0.472± 0.185	0.447± 0.096	0.409± 0.111	0.446± 0.108	0.435± 0.068	0.418± 0.135	0.436± 0.015	0.418± 0.026	0.389± 0.013	0.352± 0.011	0.405± 0.001	0.363± 0.002	0.339± 0.005	0.353± 0.026	0.374± 0.009
		Organic acids			**Furan derivates**			**Phenolic compounds**							
***S. cerevisiae* YL13-1**	**None**	**Formate**	**Acetate**	**Lactate**	**Furfural**	**5-HMF**	**Phenol**	**Benzoic acid**	**4-Hydroxy benzonic acid**	***p*-Coumaric acid**	**Vanillin**	**Vanillic acid**	**Ferulic acid**	**Syringic acid**	**Syringaldehyde**
Concentration (g/L)	/	5	8	5	0.5	0.5	0.1	0.1	0.1	0.04	0.04	0.04	0.04	0.04	0.04
OD_{600max}	13.46± 0.23	11.07± 0.16	11.07± 0.50	12.45± 0.51	12.05± 0.44	12.02± 0.23	11.98± 0.77	11.57± 0.63	11.55± 1.21	11.14± 1.14	11.95± 0.19	11.92± 0.32	11.54± 0.73	12.05± 0.18	12.35± 0.21
Ethanol concentration (g/L)	22.91± 1.23	20.28± 1.02	21.73± 0.18	22.09± 0.63	21.46± 0.98	21.51± 1.72	21.70± 0.70	20.84± 0.80	21.06± 0.15	20.89± 0.95	20.15± 0.79	20.14± 0.01	21.39± 0.40	21.85± 0.28	21.76± 1.13
Ethanol yield (g/g)	0.451± 0.024	0.399± 0.020	0.427± 0.004	0.434± 0.014	0.422± 0.019	0.423± 0.034	0.427± 0.027	0.410± 0.016	0.414± 0.003	0.411± 0.0190	0.396± 0.015	0.386± 0.000	0.421± 0.008	0.445± 0.013	0.428± 0.022
		Organic acids			**Furan derivates**			**Phenolic compounds**							

S. cerevisiae YL13-2	None	Formate	Acetate	Lactate	Furfural	5-HMF	Phenol	Benzoic acid	4-Hydroxy benzonic acid	*p*-Coumaric acid	Vanillin	Vanillic acid	Ferulic acid	Syringic acid	Syringaldehyde
Concentration (g/L)	/	5	8	5	0.5	0.5	0.1	0.1	0.1	0.04	0.04	0.04	0.04	0.04	0.04
OD_{600max}	14.02± 0.45	12.37± 0.61	12.52± 0.40	13.33± 0.73	13.27± 0.27	13.98± 1.02	12.99± 0.72	12.66± 0.39	12.22± 0.57	12.54± 0.12	12.87± 0.75	12.87± 0.75	13.48± 0.35	13.84± 0.14	13.73± 0.66
Ethanol concentration (g/L)	23.24± 0.73	21.49± 0.87	22.65± 0.18	23.66± 0.65	22.87± 0.22	23.07± 0.18	22.49± 0.22	22.18± 0.79	22.77± 0.62	21.98± 0.55	21.69± 0.55	21.02± 0.43	22.18± 0.19	22.62± 0.65	22.31± 0.49
Ethanol yield (g/g)	0.457± 0.014	0.423± 0.017	0.446± 0.004	0.465± 0.013	0.450± 0.004	0.454± 0.004	0.442± 0.024	0.436± 0.015	0.448± 0.012	0.432± 0.011	0.427± 0.011	0.413± 0.008	0.436± 0.004	0.430± 0.005	0.439± 0.010

Ethanol yield = maximum ethanol concentration in batch fermentation / the consumed sugars × 100%

Table S5. Ethanol fermentation performances by strains YL13-2 using undetoxified SECSH without adding YP and with adding YP.

	Without adding YP	With adding YP
Ethanol concentration (g/L)	20.04±0.19	20.39±0.14
Ethanol yield (g/g)	0.438±0.004	0.445±0.003
Ethanol yield (%)	85.60±0.80	87.11±0.58
Ethanol productivity (g/L h)	0.56±0.01	0.57±0.01

Table S6. Ethanol fermentation performances by different strains using undetoxified SECSH.

Strain	M3013	YL13-1	YL13-2
Residual sugar (g/L)	10.08±0.79	3.53±0.59	0±0.00
Ethanol concentration (g/L)	20.62±1.07	22.43±0.79	22.96±1.73
Ethanol yield (g/g)	0.415±0.022	0.442±0.016	0.454±0.034
Ethanol yield (%)	81.22±4.23	86.52±3.12	88.88±6.82
Ethanol productivity (g/L h)	0.57±0.03	0.61±0.02	0.63±0.05

Additional references

[1] Y.J. Li, M.M. Wang, Y.W. Chen, M. Wang, L.H. Fan, T.W. Tan, Engineered yeast with a CO_2-fixation pathway to improve the bio-ethanol production from xylose-mixed sugars, Sci Rep 7 (2017) 43875. https://doi.org/10.1038/srep43875.