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Enhanced ethanol production from sugarcane molasses by industrially engineered *Saccharomyces cerevisiae* via replacement of the *PHO4* gene

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Table S1. Primer sequences used for PCR

Gene	Forward primer (5′-3′)	Reverse primer (3′-5′)
PHO4	TTTCCAGCAAAGCGCCTCTT	GAAGTCATGCTTCGGAAGGACC

Table S2. Approaches for improved ethanol production of industrial *Saccharomyces cerevisiae* strains via genetic engineering (GE) or metabolic engineering (ME) or genome modification (GM)

Name	Details	Approach	References
UMArn3.3	Intracellular free overexpression of *ISU1* and *JAC1* (pYES2 plasmid)	GE (OE)	1
ZS*pTΔ A*	Intracellular free overexpression of *TPS1* (pUG6E plasmid) and deletion of *ATH1*	GE (OE + HR + *Δ*)	2
Sc4126z	Intracellular free expression of pRS316ZFP-M01 (artificial zinc finger protein library, AZFP)	GE (AZFP)	3
KAM-12	Intracellular integrated overexpression of *GLT1* in *gpd1* deletion (integrated to the genome based on homologous recombination)	GE (OE + HR)	4
KAM-11	Intracellular integrated overexpression of *GLT1* in *FPS1* deletion (integrated to the genome based on homologous recombination)	GE (OE + HR)	4
TS5	Deletion of *fps1* and expression of *GAPN* mutant, drug resistance marker-aided genome shuffling (with the Cre/loxP system, based on homologous recombination)	ME + GM (GS, HR)	5
IMI056	Integrated cassette (pFA6a-*TRP1*-P_{ADH1}-*SUC2*) into chromosome	ME (HR)	6
iETS2	Integrating the SPT15 mutant alleles of ETS2 by gTME into chromosome	GM (gTME + HR)	7
iETS3	Integrating the SPT15 mutant alleles of ETS3 by gTME into chromosome	GM (gTME + HR)	7

YZ1	Three rounds genome shuffling of original strains Z8 and Z15 (sporulation and hybridisation) in combination with optimised initial selection	GM (GS)	8
CAE1	Genome replication engineering-assisted continuous evolution (GREACE)	GM (GREACE)	9
M1	Error-prone PCR was employed to engineer the subunit Rpb7 of RNAP II	TE + gTME	10
ISO12	Using a long-term adaptation evolution strategy	AE	11
PHY	Yeast surface display system (YSD)	GE (YSD)	12
BY4741X/*ΔPHO13*	Deletion of the *PHO13* gene	GE (*Δ*)	13
JX123_noxE	Engineered through Cas9 (CRISPR associated protein 9)-based genome editing: auxotrophic mutants and introduced a xylose metabolic pathway into the auxotrophic mutants	ME (CRISPR/Cas9)	14
MEC1121	A novel metabolic pathway assembly tool called the Yeast Pathway Kit158 (YPK)	ME	15
P6H9	Evolutionary engineering	EE	16
FL20	Flocculation gene *FLO1* transferred into PE-2	GE	17
MF01-PHO4[b]	Replacement of *PHO4* gene	GR (HR + DTL)	This study

Note: *Δ*, deletion of gene(s); GE, genetic engineering; ME, metabolic engineering; OE, overexpression; GM, genome modification; gTME, global transcription machinery engineering; GS, genome shuffling; AZFP, artificial zinc finger protein; HR, homologous recombination; TE, transcriptional engineering; AE, adaptive evolution; YSD, yeast surface display; design-test-learn (DTL).

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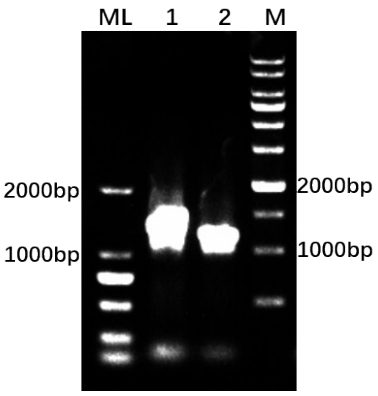

Fig. S1. Electrophoresis of *PHO4* fragment from *S. cerevisiae* MC15 on a 0.8% (w/v) agarose. Lane ML: DL2000 DNA Marker; Lane M: 1kb DNA Marker; Lane 1,2: PCR product of *PHO4* fragment from *S. cerevisiae* MC15.

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Table S3. Comparison of the amino acid composition of Pho4 protein of *S. cerevisiae* MF01-PHO4, MF01 and S288C strain

	Strain(GenBank Accession No.)					
	MF01-PHO4(MK781979)		MF01(MK781980)		S288C(NP 116692)	
Amino Acid	Frequency	Percentage （%）	Frequency	Percentage （%）	Frequency	Percentage （%）
Ala (A)	26	8.3	26	8.3	26	8.3
Arg (R)	17	5.4	18	5.8	18	5.8
Asn (N)	19	6.1	19	6.1	19	6.1
Asp (D)	18	5.8	18	5.8	18	5.8
Cys (C)	1	0.3	1	0.3	1	0.3
Gln (Q)	10	3.2	10	3.2	10	3.2
Glu (E)	20	6.4	20	6.4	20	6.4
Gly (G)	13	4.2	12	3.8	12	3.8
His (H)	13	4.2	13	4.2	13	4.2
Ile (I)	11	3.5	12	3.8	11	3.5
Leu (L)	22	7.1	22	7.1	22	7.1
Lys (K)	21	6.7	20	6.4	20	6.4
Met (M)	5	1.6	5	1.6	5	1.6
Phe (F)	5	1.6	5	1.6	5	1.6
Pro (P)	23	7.4	23	7.4	23	7.4
Ser (S)	40	12.8	40	12.8	40	12.8
Thr (T)	22	7.1	22	7.1	22	7.1
Trp (W)	2	0.6	2	0.6	2	0.6
Tyr (Y)	4	1.3	4	1.3	4	1.3
Val (V)	20	6.4	20	6.4	21	6.7

Table S4. Comparison of characteristics of Pho4 protein of *S. cerevisiae* MF01-PHO4, MF01 and S288C strain

Strain	Formula	Total number of atoms	Molecular weight	Theoretical Isoelectric point (pI)	Aliphatic index	Instability index
MF01-PHO4	$C_{1460}H_{2351}N_{441}O_{484}S_6$	4742	34018.75	7.25	68.17	58.79
MF01	$C_{1464}H_{2359}N_{443}O_{484}S_6$	4756	34102.87	7.25	69.42	61.11
S288C	$C_{1463}H_{2357}N_{443}O_{484}S_6$	4753	34089.30	7.95	65.85	61.01

Note: references to the website(1) SGD: https://www.yeastgenome.org/locus/S000001930/protein, (2) Expasy：http://web.expasy.org/protparam/

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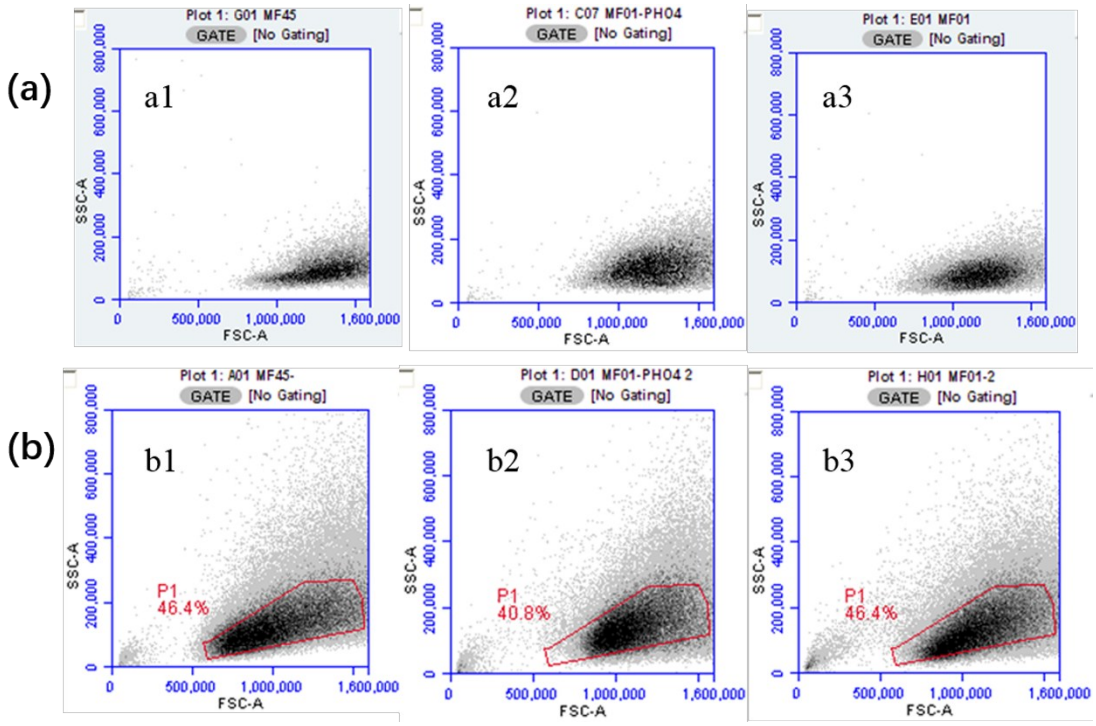

Fig. S2. Flow cytometric results (FSC/SSC) of *S. cerevisiae* MC15, MF01-PHO4 and MF01 strain; (a) Cultivated in YPD at 30°C, shaking at 200 rpm for 8 h; (B) Cultivated in YPS40 at 30°C, shaking at 180 rpm for 48 h; a1 or b1, *S. cerevisiae* MC15 strain; a2 or b2, recombinant *S. cerevisiae* MF01-PHO4 strain; a3 or b3, *S. cerevisiae* MF01 strain

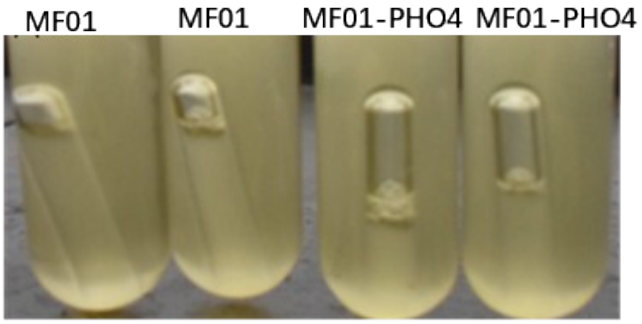

Fig. S3 Gas production from *S. cerevisiae* MF01 and engineered MF01-PHO4 strain

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Table S5. Summary of ethanol production from SCM by *S. cerevisiae* strains

Name	Tm (℃)	Production (g L^{-1})	Yield ($g_{Ethanol}$ /g_{sugar})	Fermentation Efficiency (%)	pH	References
ITV-01	30	85.00	0.4100	80.39	5.5	18
Illovo	30	87.00	0.4341	85.12	----	19
M Type	32	82.17	----	----	----	20
CAT-1	30-32	79.25	----	----	----	21
CAT-1	30	86.82	----	----	----	21
PE-2	30-32	66.06	0.4700	92.16	----	21
PE-2	35	72.62	----	----	----	22
ΔRIM15ΔMS	35	77.35	----	----	----	22
VR1	30-32	50.91	----	----	----	21
BG1	30-32	59.28	0.4900	96.08	----	21
JP1	30-32	56.20	0.4700	92.16	----	23
UAF-1	31-33	96.29	----	----	4.0-4.5	24
y7	28-35	68.35	0.3817	74.85	6	25
F396	28-35	68.98	----	----	6	25
345	28-35	70.41	----	----	6	25
Rasse XII	28-35	72.77	0.3917	76.80	6	25
109	28-35	72.93	----	----	6	25
115	28-35	68.75	----	----	6	25
116	28-35	69.85	----	----	6	25
Rlle IID	28-35	67.25	----	----	6	25
Sacch.Sake	28-35	69.54	----	----	6	25
MF02	30	114.29	----	----	3.9	26
MF03	30	106.56	----	----	3.9	26
AQ01	30	89.19	----	----	3.9	26
AQ02	30	88.88	----	----	3.9	26
NF-ybr	30	98.82	----	----	3.9	27
MF01[a]	30	108.61	0.4789	93.90	3.6-3.8	28
100-294	37	73.0	----	----	5.5	29
AQ01	37	86.35	----	----	3.9	26
AQ02	37	85.80	----	----	3.9	26
MF02	37	95.19	----	----	3.9	26
MF03	37	95.82	----	----	3.9	26
MF01	37	94.95	----	----	3.9	26

MC15	30	82.16 ± 0.79	0.3510	68.83	3.8	This study
MF01	30	108.94 ± 0.71	0.4654	91.26	3.8	This study
MF01-PHO4	30	114.71 ± 0.24	0.4901	96.10	3.8	This study

Note: [a]annual production of 50,000 tonnes of ethanol from SCM fermentation

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