

Supporting Information for
Improving heterologous expression of laccase by *Pichia pastoris*
via vanillin-induced stress response and its application for
removing inhibitors of lignocellulose hydrolysate

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Figure S1. Single clone of the recombinant strain PM(n)-2*gfp*-pPIC9K-*P. pastoris* GS115 with alternative promoter combined with 2GFP on MD plate.

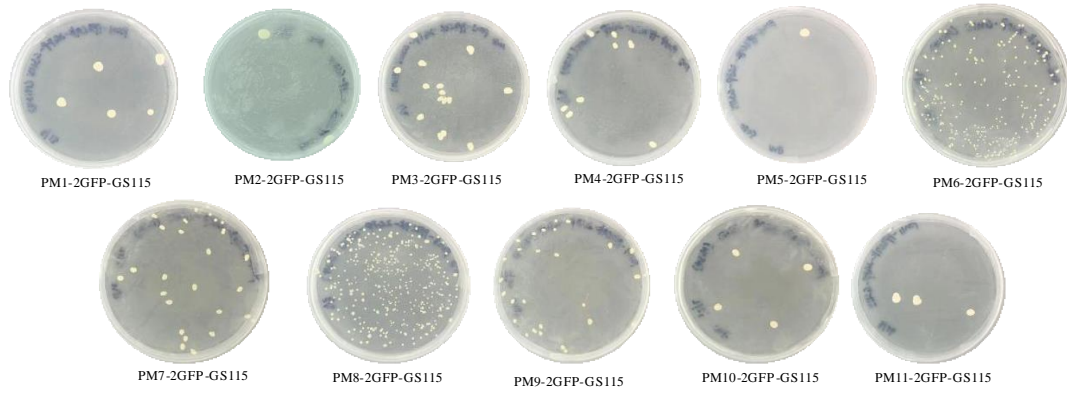
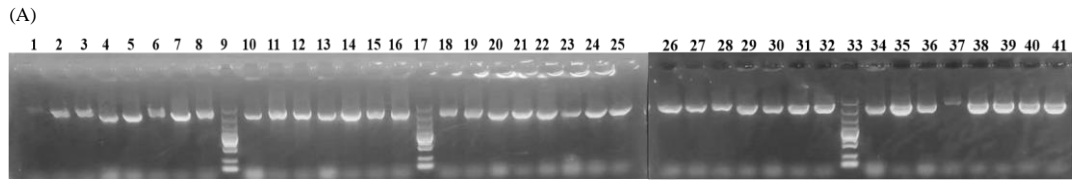
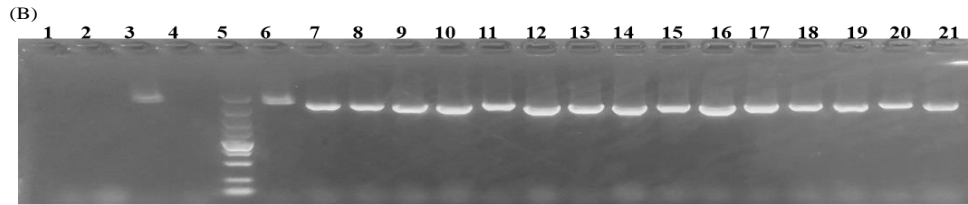


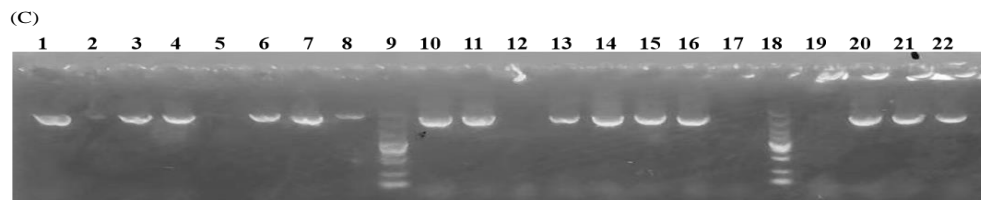
Figure S2. Colony PCR of each single clone of the recombinant strain PM(n)-2gfp-pPIC9K-*P. pastoris* GS115.



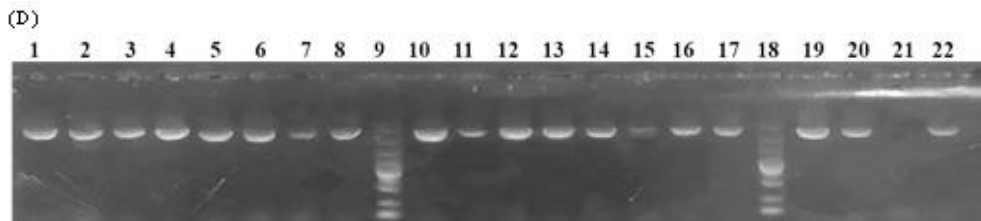
Lane1~Lane16: No.1~No.15 single clone of PM(8)-2gfp-pPIC9K-*P. pastoris* GS115;
Lane18~Lane32: No.1~No.15 single clone of PM(3)-2gfp-pPIC9K-*P. pastoris* GS115;
Lane34~Lane41: No.1~No.8 single clone of PM(4)-2gfp-pPIC9K-*P. pastoris* GS115;
The target length of each fragment is 3173bp; Lane9/Lane17/Lane33: Takara DL5K



Lane1~Lane4: No.1~No.4 single clone of PM(10)-2gfp-pPIC9K-*P. pastoris* GS115
(Target length of each fragment is 3348bp); Lane6~Lane21: No.1~No.16 single clone
of PM(6)-2gfp-pPIC9K-*P. pastoris* GS115 (Target length of each fragment is 3173bp);
Lane5: Takara DL5K.

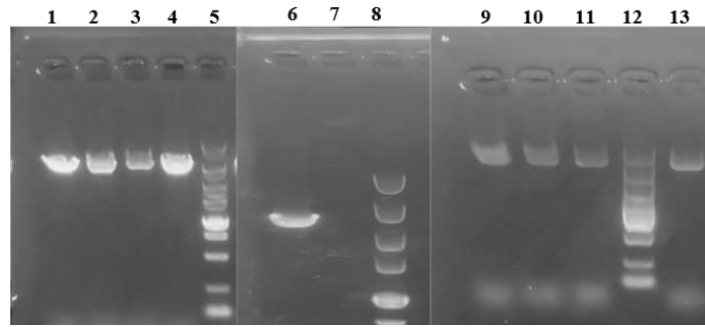


Lane1~Lane22: No.1~No.4 single clone of PM(7)-2gfp-pPIC9K-*P. pastoris* GS115
(Target length of each fragment is 3348bp); Lane9/Lane18: Takara DL5K



Lane1~Lane22: No.1~No.4 single clone of PM(9)-2gfp-pPIC9K-*P. pastoris* GS115
(Target length of each fragment is 3348bp); Lane9/Lane18: Takara DL5K

(E)



Lane1~Lane4: No.1~No.4 single clone of PM(1)-2gfp-pPIC9K-*P. pastoris* GS115;

Lane6~Lane7: No.1~No.2 single clone of PM(2)-2gfp-pPIC9K-*P. pastoris* GS115;

Lane9~Lane11: No.1~No.3 single clone of PM(11)-2gfp-pPIC9K-*P. pastoris* GS115;

Lane13: single clone of PM(5)-2gfp-pPIC9K-*P. pastoris* GS115 (Target length of each fragment is 3348bp); Lane5/Lane8/Lane12: Takara DL5K

Figure S3. Plasmid map of vanillin-sensitive promoters PM4 and PM7 recombined with *lacc6* gene respectively. (A) PM(4)-*lacc6*-P_{AOX}-*lacc6*-pPIC9K; (B) PM(4)-P_{AOX}-*lacc6*-pPIC9K; (C) PM(4)-*lacc6*-pPIC9K; (D) PM(7)-*lacc6*-P_{AOX}-*lacc6*-pPIC9K; (E) PM(7)-P_{AOX}-*lacc6*-pPIC9K; (F) PM(7)-*lacc6*-pPIC9K;

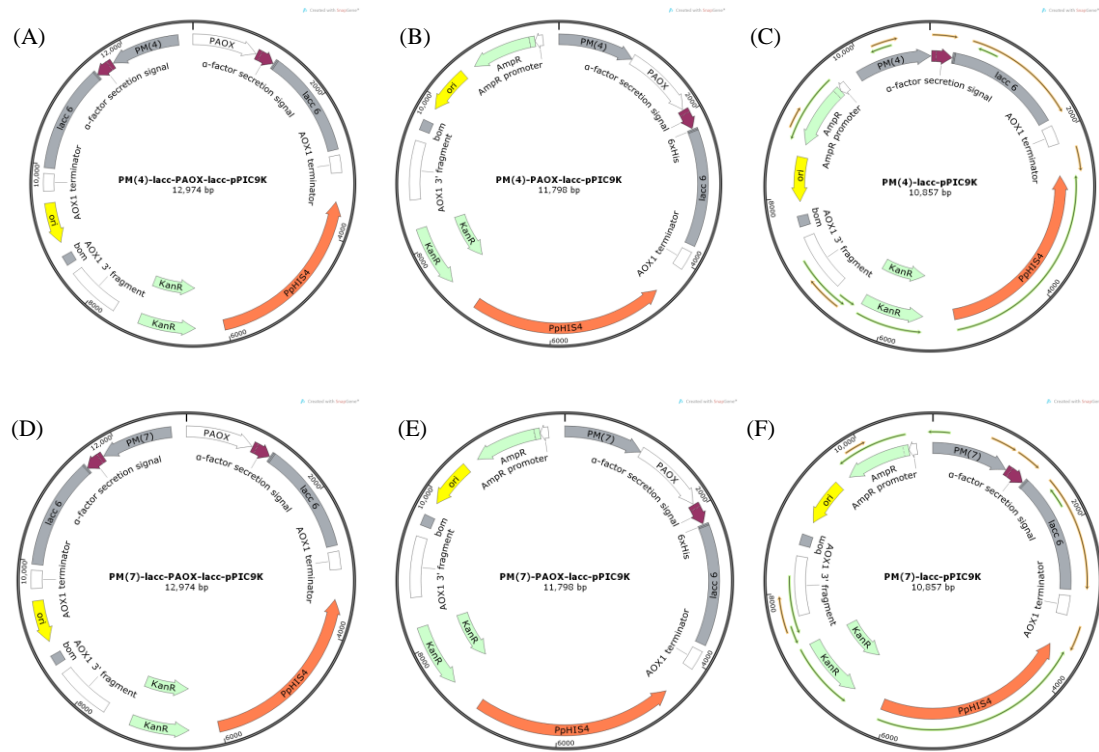
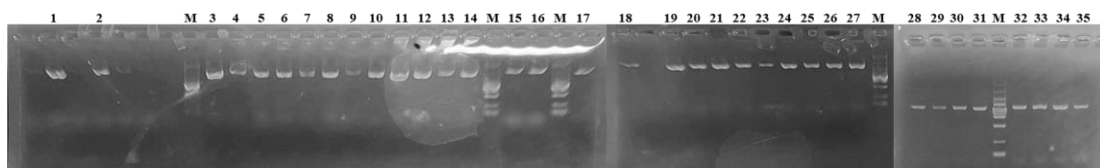


Figure S4. Colony PCR of each single clone of vanillin sensitive promoter and laccase gene recombinant strains



Lane1~Lane2: No.1~No.2 single clone of PM(4)-P_{AOX}-*lacc*-pPIC9K-*P. pastoris* GS115 (Target fragment length is 2538 bp); Lane3~Lane14: No.1~No.12 single clone of PM(4)-*lacc*-pPIC9K-*P. pastoris* GS115 (Target fragment length is 2108 bp); Lane15~Lane16: No.1~No.2 single clone of PM(7)-P_{AOX}-*lacc*-pPIC9K-*P. pastoris* GS115 (Target fragment length is 2539 bp); Lane17~Lane27: No.1~No.11 single clone of PM(7)-*lacc*-pPIC9K-*P. pastoris* GS115 (Target fragment length is 2113 bp); Lane28~Lane31: No.1~No.4 single clone of PM(4)-*lacc*-P_{AOX}-*lacc*-pPIC9K-*P. pastoris* GS115 (Target fragment length is 1041bp); Lane32~Lane35: No.1~No.4 single clone of PM(7)-*lacc*-P_{AOX}-*lacc*-pPIC9K-*P. pastoris* GS115 (Target fragment length is 1038bp); M: Takara DL5K

Figure S5. FPKM value for *lacc6* in PM(4)-P_{AOX}-2*lacc*-GS115-1 and P_{AOX}-*lacc*-GS115 PM4_Van: PM(4)-P_{AOX}-2*lacc*-GS115-1 cultured with vanillin, PM4: PM(4)-P_{AOX}-2*lacc*-GS115-1 cultured without vanillin, PAOX_Van: P_{AOX}-*lacc*-GS115 cultured with vanillin, PAOX: P_{AOX}-*lacc*-GS115 cultured without vanillin

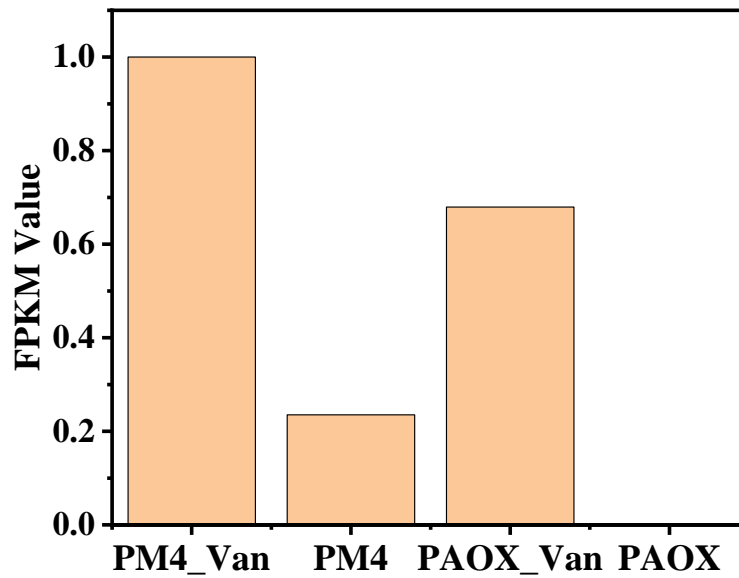


Figure S6. Change of vanillin concentration before and after fermentation by PM(4)-P_{AOX}-2*lacc*-GS115-1. (A) Comparison on the UV spectra of vanillin and fermentation broth (without vanillin); (B) Change of vanillin concentration in fermentation broth with time for experimental group (addition of vanillin and methanol to induce laccase expression) and control group (without methanol); (C) UV-spectrum of fermentation broth supernatant at 0 h and 48 h for experimental group; and (D) UV-spectrum of fermentation broth supernatant at 0 h and 48 h for control group.

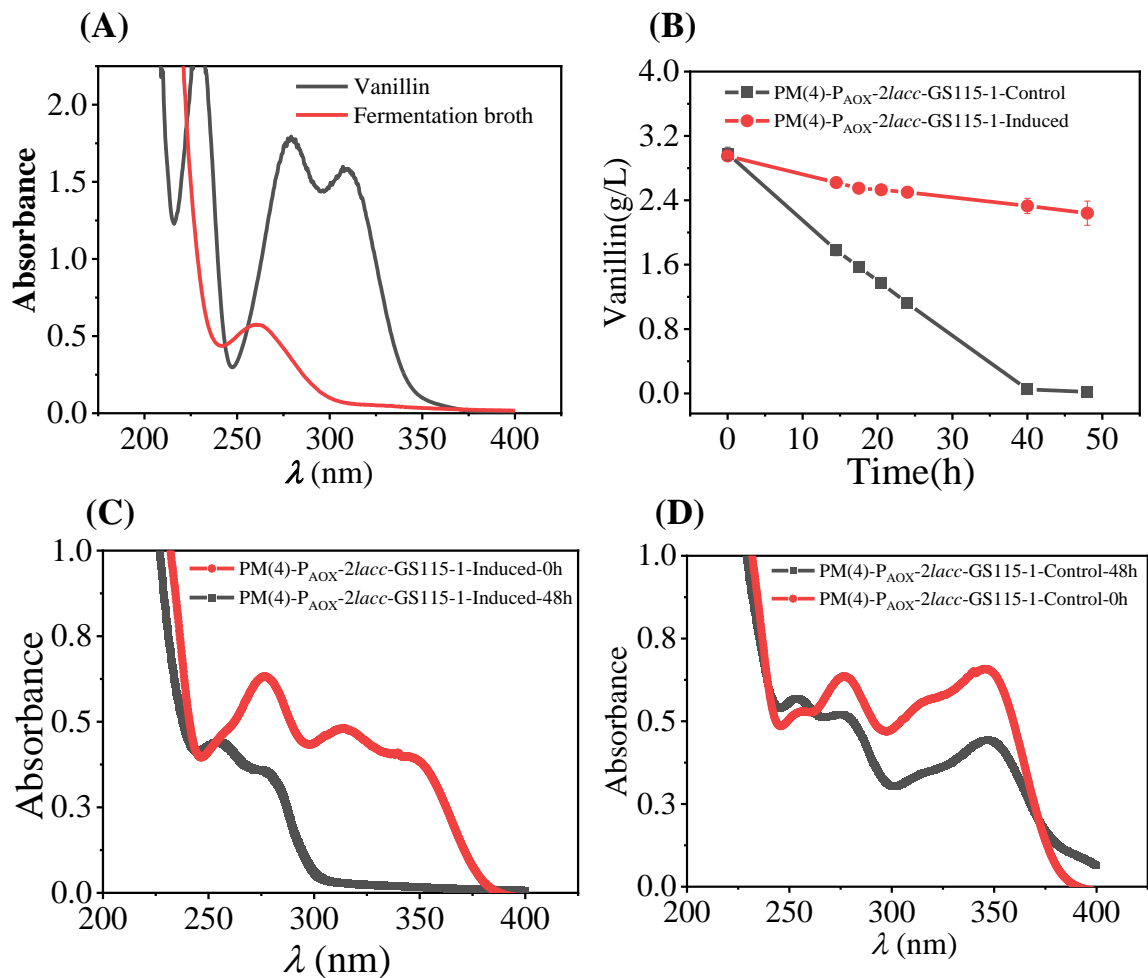


Figure S7. Addition of laccase in enzymatic hydrolysis of microcrystal cellulose (Avicel) with and without presence of lignin or lignin model compounds. (A) Klason lignin; (B) Tannic acid as a lignin model compound.

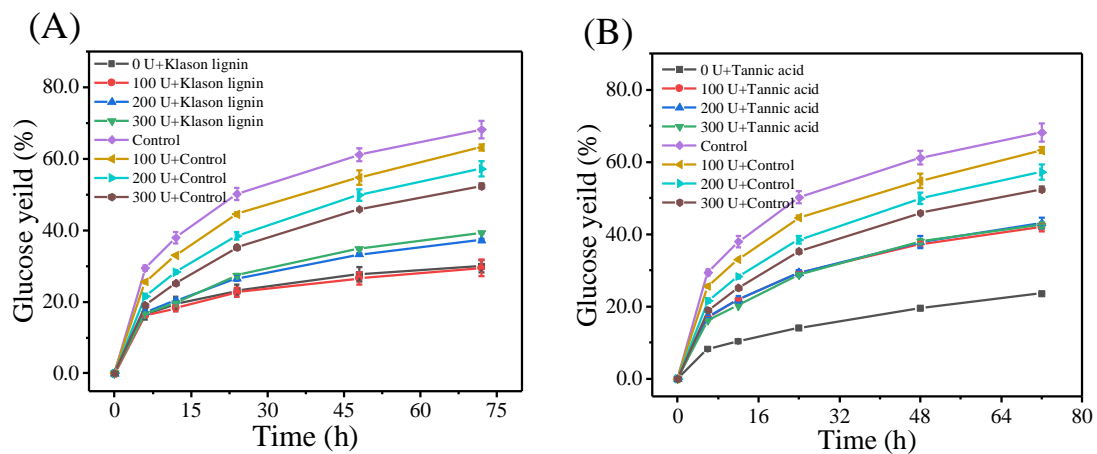


Figure S8. Change of phenolic and furan aldehyde stress factors under non-discharging conditions in 1.5 M KOH. (A) Vanillin; (B) Syringaldehyde; (C) *p*-hydroxy benzaldehyde; (D) Furfural; (E) 5-hydroxymethylfurfural

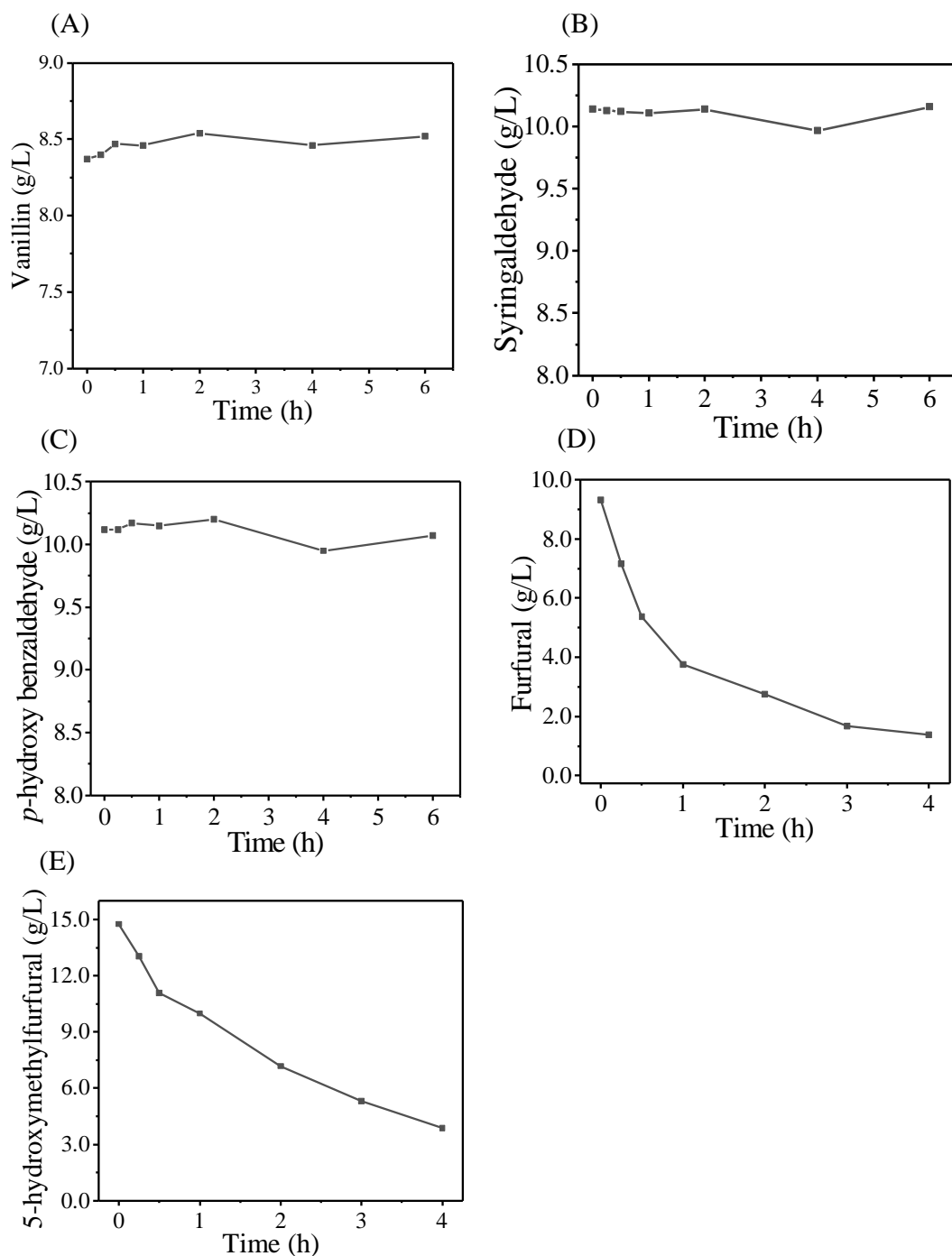


Table S1 Primers of PM(n)-2*gfp*-pPIC9K construction

Plasmid	Primer	Sequence
PM1-2 <i>gfp</i> -pPIC9 K	PM1-F	ACAGTTATTATTCGACGATGACCACCTTGGTAGAGGA
	PM1-R	TCATCGTTTGGATCCCTACCAGGGCTTTTCTTAACTTATATATCCTGG
	PM1V-F	GAAAAGCCCTGGTAGGGATCCAAACGATGAGATTCCTTCAATT
	PM1V-R	CCAAGGTGGTCATCGTCGAATAATAACTGTTATTTTCAGTGTCCCGATCTG
PM2-2 <i>gfp</i> -pPIC9 K	PM2-F	ACAGTTATTATTCGACCCTCATATACATAAATAGATAGCACCACGAT
	PM2-R	TCATCGTTTGGATCCTGGGTTGATCCTACGGAAAACGTC
	PM2V-F	CGTAGGATCAACCCAGGATCCAAACGATGAGATTCCTTCA
	PM2V-R	TTATGTATATGAGGGTCGAATAATAACTGTTATTTTCAGTGTCCCGATCTG
PM3-2 <i>gfp</i> -pPIC9 K	PM3-F	TCATCGTTTGGATCCTTCAAGTCAACTTTTCAAGTGATCCAACTGT
	PM3-R	ACAGTTATTATTCGAAATTGGTTTGCCTGTTGTTTCAAATTGT
	PM3V-F	AAAAGTTGACTTGAAGGATCCAAACGATGAGATTCCTTCAA
	PM3V-R	ACAGGCAAACCAATTTTGAATAATAACTGTTATTTTCAGTGTCCCGAT
PM4-2 <i>gfp</i> -pPIC9 K	PM4-F	TCATCGTTTGGATCCGATGAGTTACAGAGAGTACGAAAAAGAAGAGAGAG
	PM4-R	ACAGTTATTATTCGACCAAACCTTATGGAAGAACTTGGATACAGC
	PM4V-F	TCTCTGTAACCTCATCGGATCCAAACGATGAGATTCCTTCAATTTT
	PM4V-R	CTTCATAAGTTTGGTCGAATAATAACTGTTATTTTCAGTGTCCCGATCTG
PM5-2 <i>gfp</i> -pPIC9 K	PM5-F	TCATCGTTTGGATCCTTCAAAGTGTTTATGATTGAGGTTCAAGACAG
	PM5-R	ACAGTTATTATTCGAACATATGATTGGTGCCTACGTTTTGC
	PMV5-F	CATAAACACTTTGAAGGATCCAAACGATGAGATTCCTTCAA
	PMV5-R	GCACCAATCATATGTTTGAATAATAACTGTTATTTTCAGTGTCCCGAT
PM6-2 <i>gfp</i> -pPIC9 K	PM6-F	ACAGTTATTATTCGATTGTACAAAGATTGGGCGTCCAAG
	PM6-R	CTCATCGTTTGGATCCGTTGCTGTTGGAGGTAAAAACTTTAGAGAATATG
	PM6V-R	CCAATCTTTGTACAATCGAATAATAACTGTTATTTTCAGTGTCCCGAT
	PMV6-F	TACCTCCAACAGCAACGGATCCAAACGATGAGATTCCTTCAATTTT
PM7-2 <i>gfp</i> -pPIC9 K	PM7-F	ACAGTTATTATTCGATTGCAGGCAAATAAGTCAACCACG
	PM7-R	TCATCGTTTGGATCCACCTAAGTCATATAGGTGGTGTCTCTCG
	PM7V-F	CTATATGACTTAGGTGGATCCAAACGATGAGATTCCTTCAATT

	PM7V-R	TTATTTTGCCTGCAATCGAATAATAACTGTTATTTTTCAGTGTTCCCGAT
PM8-2 <i>gfp</i> -pPIC9 K	PM8-F	AAATCTCATCGTTTGGATCCGGGGAAACCTCCACTATTTATATACATGTCC
	PM8-R	ACAGTTATTATTTCGAATGATACCACAAAGATGAAAGTTTATGCCAAAC
	PM8V-F	TAAATAGTGGAGGTTTCCCCGGATCCAAACGATGAGATTTCTTCAATTTT
	PM8V-R	TCTTTGTGGTATCATTTCGAATAATAACTGTTATTTTTCAGTGTTCCCGAT
PM9-2 <i>gfp</i> -pPIC9 K	PM9-F	ACAGTTATTATTTCGACTAAAAAAGTTTGAAATGCTGAAGCGGAAA
	PM9-R	TCATCGTTTGGATCCATTATGATTTGTGAGGTGAAAGTGAATGTTTCTATTT
	PM9V-F	CTCACAAATCATAATGGATCCAAACGATGAGATTTCTTCAATT
	PM9V-R	TCAAACTTTTTTTAGTCGAATAATAACTGTTATTTTTCAGTGTTCCCGATCTG
PM10-2 <i>gfp</i> -pPIC9 K	PM10-F	TCATCGTTTGGATCCTAAGAGTTAATTCGATAGTAAGAGGGATCAGCAAG
	PM10-R	ACAGTTATTATTTCGACACGCCTAAGCTGAAGCTTCC
	PM10V-F	TCAATTAACCTTAGGATCCAAACGATGAGATTTCTTCA
	PM10V-R	TTCAGCTTAGGCGTGTGCAATAATAACTGTTATTTTTCAGTGTTCCCGATCTG
PM11-2 <i>gfp</i> -pPIC9 K	PM11-F	TCATCGTTTGGATCCTCAGGTGGATCAAGTCTATCAGTTGAAATTCT
	PM11-R	ACAGTTATTATTTCGATACTGTTTGAACGTAACGAGCTTGG
	PM11V-F	ACTTGATCCACCTGAGGATCCAAACGATGAGATTTCTTCA
	PM11V-R	TACGTTCAAACAGTATCGAATAATAACTGTTATTTTTCAGTGTTCCCGAT

Table S2 Primers of PM(n)-*lacc*-pPIC9K construction

Plasmid	Primer	Sequence
P _{AOX} - <i>lacc</i> -pPIC9K	nPloc-V-R	CCGCCTCCTCCGTGGTGGTGGTGGTGGTGCATCTACGTAAGCTTCAGCC TCT
	nPloc-V-F	CATCAAAGCTGCTTATGGGCTAGGCGAATTAATTCGCCTTAGACAT
	nPloc-F	ACCACCACCACCACGGAGGAGGCGGTAGCGCTATCGGTCCAGTCGGAA
	nPloc-R	GTCTAAGGCGAATTAATTCGCCTAGCCCATAAAGCAGCTTTGATGGGT
PM(4)-P _{AOX} -2 <i>lacc</i> - pPIC9K	4-2A-F	GTA ACTCATCGGATCCAAACGATGAGATTTCTTCAATTT
	4-2A-R	ATGAGTAAACTTGGTCTGACAGTCTCACTTAATCTTCTGTACTC
	4-2F	CAC TTTTCGGGGAAATGTGCCAAACTTATGGAAGAACTTGGA
	4-2R	AATCTCATCGTTTGGATCCGATGAGTTACAGAGAGTACGA
	4-2V-F	AAGTTCCTCCATAAGTTTGGCACATTTCCCCGAAAAGTGCCA
	4-2V-R	AGAGTACAGAAGATTAAGTGAGACTGTCAGACCAAGTTTACTCAT
PM(4)-P _{AOX} - <i>lacc</i> -p PIC9K	4-F	AAAATAACAGTTATTATTCGACCAAACCTTATGGAAGAACTTGGA
	4-R	TCGTCTTTGGATGTTAGATCGATGAGTTACAGAGAGTACG
	4-V-F	TCGTACTCTCTGTA ACTCATCGATCTAACATCCAAAGACGAAAG
	4-V-R	TCCAAGTTCTTCCATAAGTTTGGTTCGAATAATAACTGTTATTTT
PM(4)- <i>lacc</i> -pPIC9 K	4P-V-F	TCAAAGCTGCTTATGGGCTAGGAATTAATTCGCCTTAGACATGAC
	4P-V-R	GGAAATCTCATCGTTTGGATCCGATGAGTTACAGAGAGTACGAA
	4Ploc-F	TGTA ACTCATCGGATCCAAACGATGAGATTTCTTCAATTTTFTA
	4Ploc-R	TCTAAGGCGAATTAATTCCTAGCCCATAAAGCAGCTTTGAT
PM(7)-P _{AOX} -2 <i>lacc</i> - pPIC9K	7-2A-F	CACCACCTATATGACTTAGGTGGATCCAAACGATGAGATTTCC
	7-2A-R	GAGTAAACTTGGTCTGACAGTCTCACTTAATCTTCTGTACTC
	7-2F	ACTTTTCGGGGAAATGTGTTGCAGGCAAAATAAGTCAACC
	7-2R	AAATCTCATCGTTTGGATCCACCTAAGTCATATAGGTGGTGTT
	7-2V-F	TTGACTTATTTTGCCTGCAACACATTTCCCCGAAAAGTGCCA
	7-2V-R	GTACAGAAGATTAAGTGAGACTGTCAGACCAAGTTTACTCAT
PM(7)-P _{AOX} - <i>lacc</i> -p PIC9K	7-F	AAAATAACAGTTATTATTCGATTGCAGGCAAAATAAGTCAACC
	7-R	TTCGTCCTTTGGATGTTAGATCACCTAAGTCATATAGGTGGTGTT
	7-V-F	CACCACCTATATGACTTAGGTGATCTAACATCCAAAGACGAAA
	7-V-R	TTGACTTATTTTGCCTGCAATCGAATAATAACTGTTATTTTTCAG
PM(7)- <i>lacc</i> -pPIC9 K	7P-V-F	AAGCTGCTTATGGGCTAGGAATTAATTCGCCTTAGACATGAC
	7P-V-R	AATCTCATCGTTTGGATCCACCTAAGTCATATAGGTGGTGTTTC
	7Ploc-F	ACACCACCTATATGACTTAGGTGGATCCAAACGATGAGATTTCTT
	7Ploc-R	ATGTCTAAGGCGAATTAATTCCTAGCCCATAAAGCAGCTTTGA

Table S3 Screened candidate promoters

Promoter	Promoter source gene ID	Promoter source gene symbol	Encoded protein
PM1	8197957	PAS_chr1-3_0024	Succinate semialdehyde dehydrogenase Deoxyhypusine hydroxylase, a HEAT-repeat
PM2	8197992	PAS_chr2-1_0553	containing metalloenzyme that catalyses hypusine formation
PM3	8198120	PAS_chr2-1_0517	Nuclear protein that contains a SET-domain
PM4	8198188	PAS_chr2-2_0288	Arginase, responsible for arginine degradation
PM5	8198636	PAS_chr2-2_0476	hypothetical protein
PM6	8198683	PAS_chr2-2_0279	Hypothetical protein
PM7	8198872	PAS_chr2-1_0404	Transcriptional repressor and activator
PM8	8198933	PAS_chr2-1_0580	Cytosolic NADP-specific isocitrate dehydrogenase
PM9	8199862	PAS_chr3_0493	Hypothetical protein
PM10	8201384	PAS_chr4_0677	Gamma-aminobutyrate (GABA) transaminase (4-aminobutyrate aminotransferase)
PM11	8201408	PAS_chr4_0460	Nonessential protein that binds actin filaments and localizes to actin patches and cables