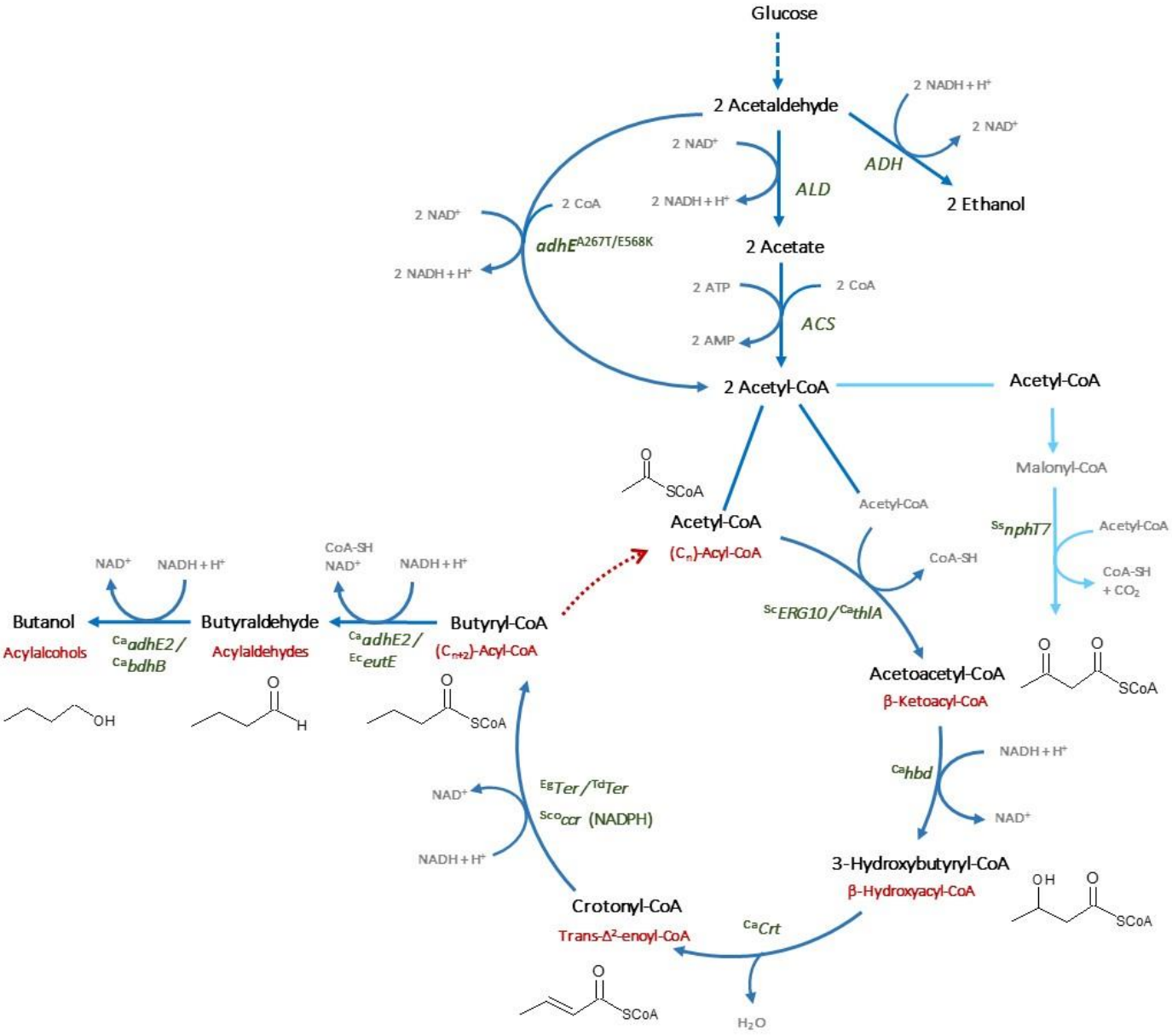

Figure S1: Metabolic pathway for n-butanol production via reverse β-oxidation in yeast.
n-Butanol production and reverse β-oxidation are shown as a process of one or several rotations, respectively, of an acetoacetyl-CoA derived synthesis pathway. This pathway can generate various compounds with different chain lengths. The different isoenzymes, tested in the former study [1], are shown for each reaction. The reaction of ^{Ss}*nphT7* (use of malonyl-CoA and acetyl-CoA for production of acetoacetyl-CoA) is shown in light blue [1].

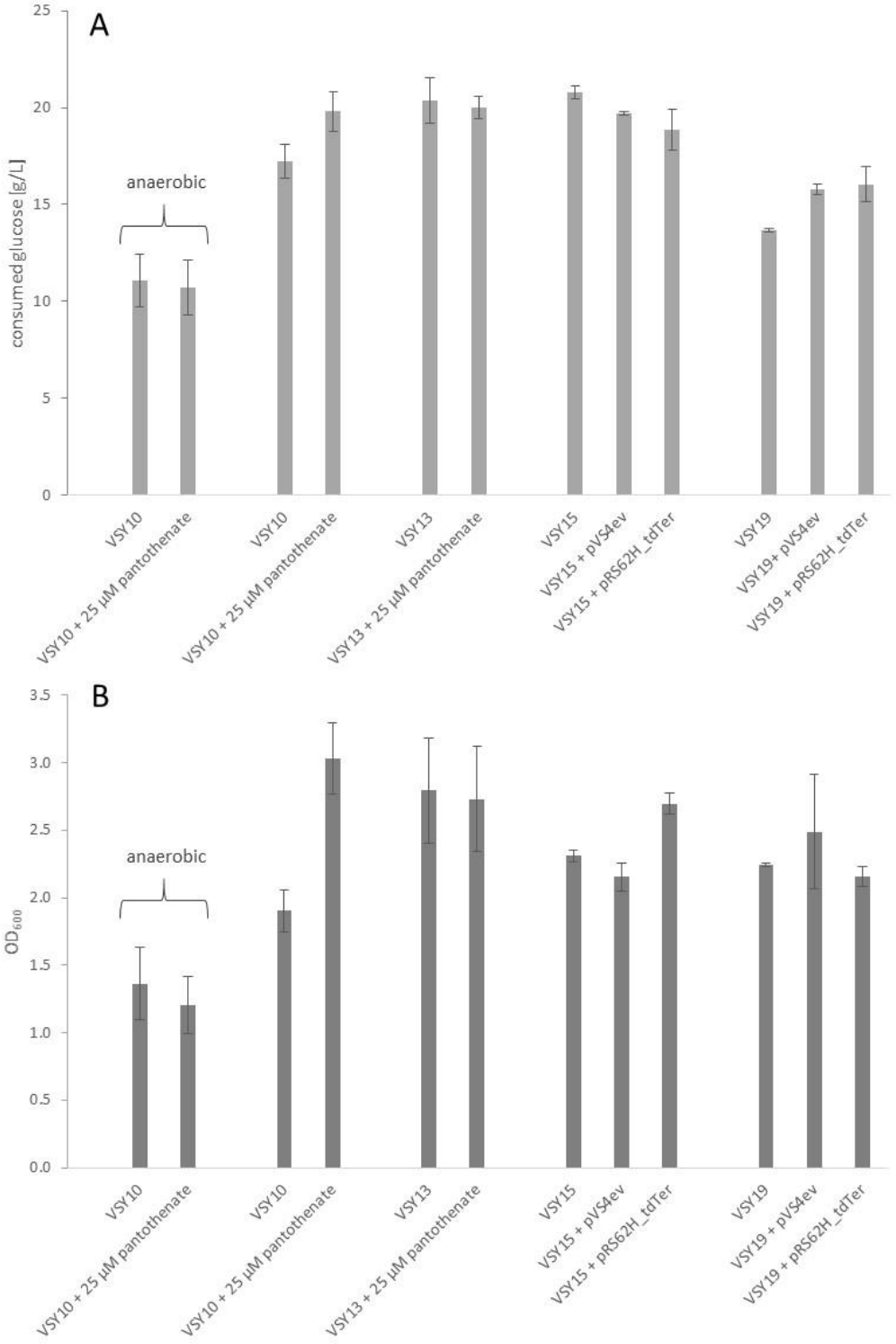

Figure S2: Comparison of glucose consumption and final OD_{600} of n-butanol production strains.
A Consumed glucose after 171 h of fermentation started with 20 g/L glucose in SMD medium. **B** The OD_{600} after 171 h of fermentations started with an OD_{600} of 0.3 in SMD medium.
Compared are fermentations of VSY10 (Δ*adh1-6 sfa1 gpd2* with n-butanol pathway genes, *coaA* and *adhE*$^{A267T/E568K}$) anaerobically and aerobically, VSY13 (like VSY10, but *adhE*$^{A267T/E568K/R577S}$ instead of *adhE*$^{A267T/E568K}$), VSY15 (VSY13 with pADH1_*FMS1*) and VSY19 (VSY15 with Δ*ald6*) with or without addition of 25 µM pantothenate or *adhE*$^{A267T/E568K/R577S}$ (pVS4ev) or pRS62H_tdTer overexpression. Error bars represent the standard deviation of three independent replicates.

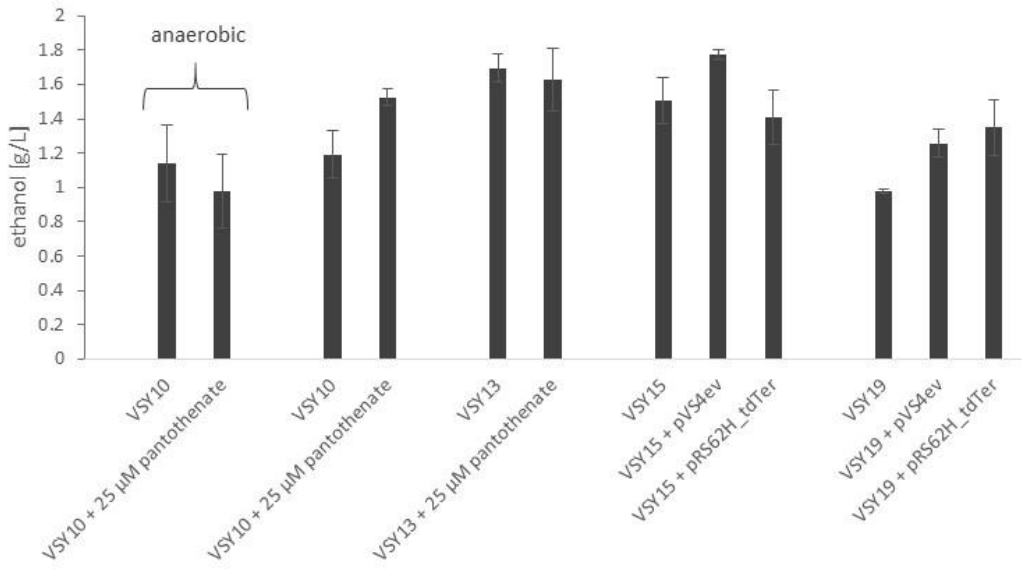

Figure S3: Comparison of ethanol concentration of n-butanol production strains.
The ethanol concentration after 171 h of fermentations started with an OD_{600} of 0.3 in SMD medium is shown. Compared are fermentations of VSY10 (Δ*adh1-6 sfa1 gpd2* with n-butanol pathway genes, *coaA* and *adhE*$^{A267T/E568K}$) anaerobically and aerobically, VSY13 (like VSY10, but *adhE*$^{A267T/E568K/R577S}$ instead of *adhE*$^{A267T/E568K}$), VSY15 (VSY13 with pADH1_*FMS1*) and VSY19 (VSY15 with Δ*ald6*) with or without addition of 25 µM pantothenate or *adhE*$^{A267T/E568K/R577S}$ (pVS4ev) or pRS62H_tdTer overexpression. Error bars represent the standard deviation of three independent replicates.

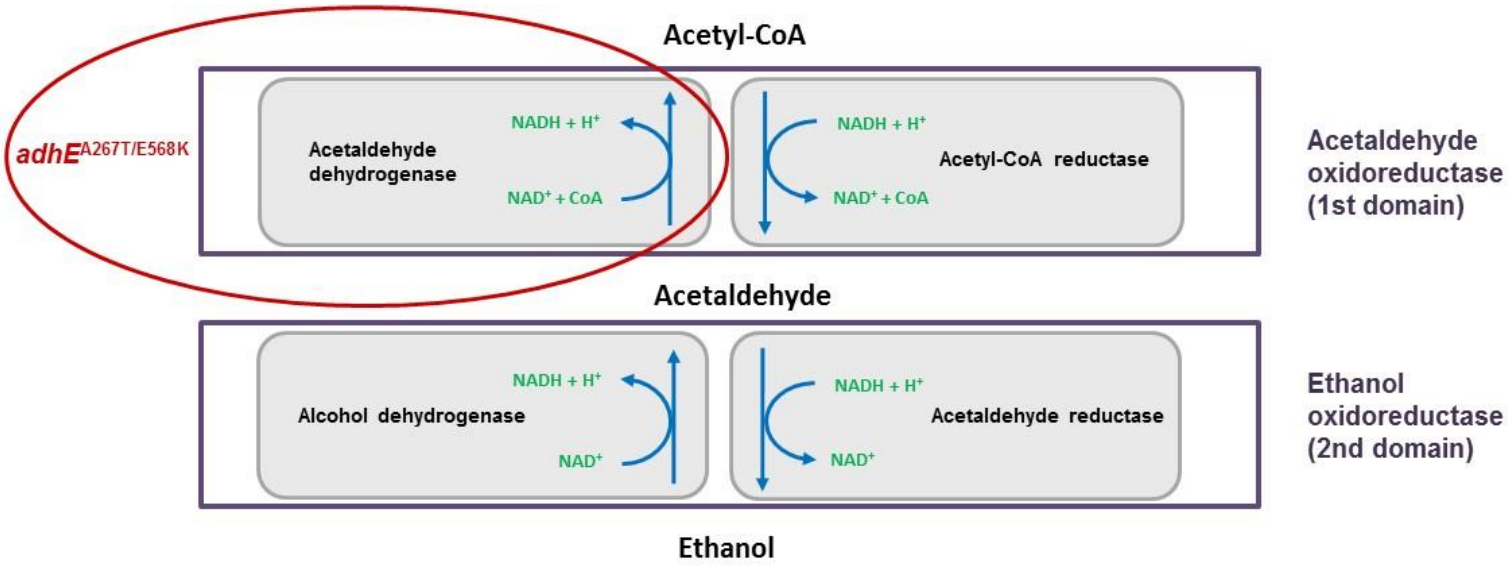

Figure S4: Enzymatic reactions of adhE from *E.coli*
Shown are possible reaction steps of multifunctional oxidoreductase adhE. With the wild type enzyme reduction from acetyl-CoA into acetaldehyde into ethanol takes place, but with amino acid changes A267T and E568K, adhE preferably converts acetaldehyde into acetyl-CoA [2].

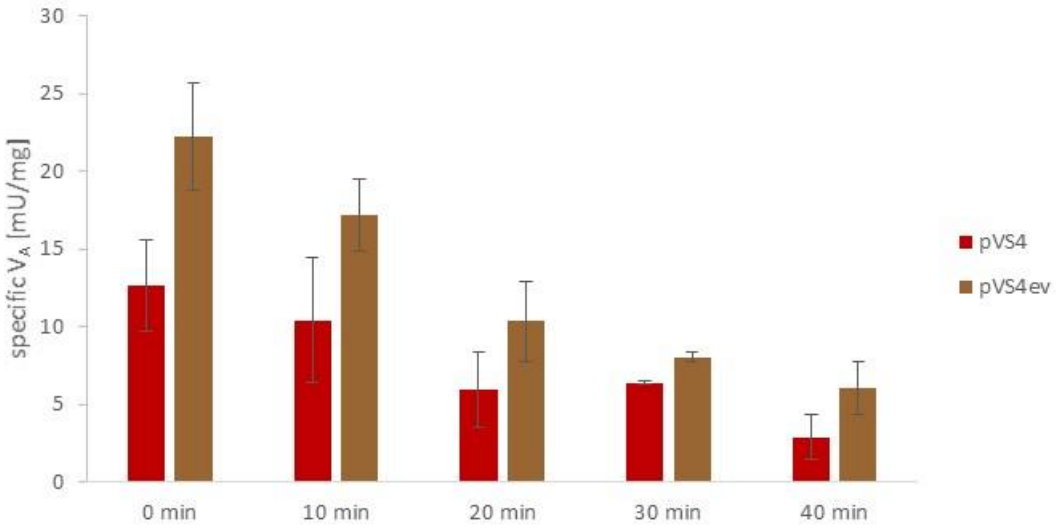

Figure S5: Stability assay of adhE$^{A267T/E568K/R577S}$.
Dehydrogenase activity of acetylating acetaldehyde dehydrogenase *adhE*$^{A267T/E568K}$ (pVS4) and *adhE*$^{A267T/E568K\ /R577S}$ (pVS4ev) was measured directly after incubation of cell extract for 0 to 40 min at 30° (based on [2]). One unit (U) is defined as the conversion of 1 µmol of substrate into the corresponding product per 1 min. The mean values of three independent replicates are shown with standard deviations.

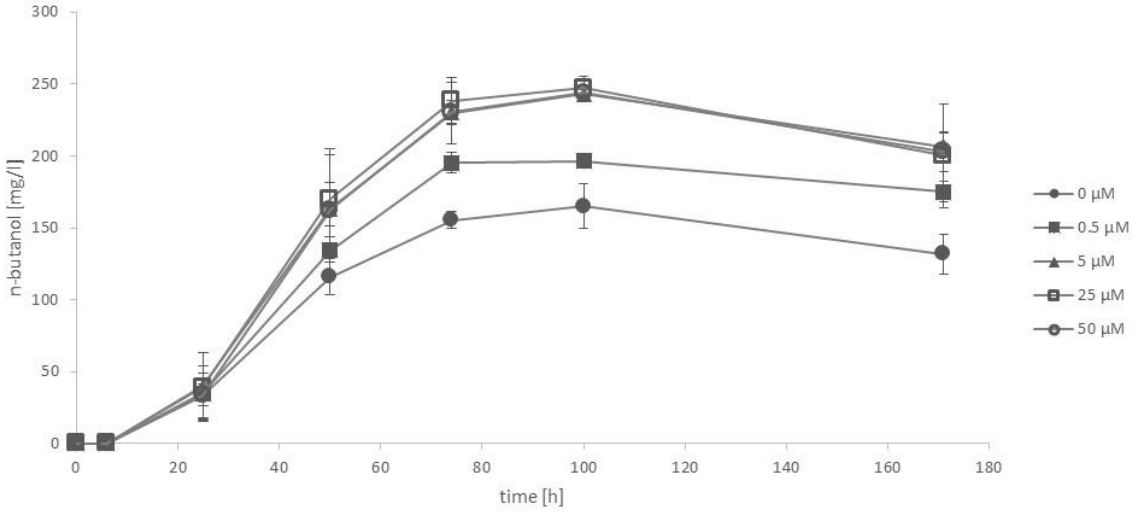

Figure S6: n-Butanol production of VSY13 under aerobic conditions with addition of pantothenate.
Five different concentrations of pantothenate were added to SMD medium: 0 µM (circle), 0.5 µM (square), 5 µM (triangle), 25 µM (open square), 50 µM (open circle). Error bars represent the standard deviation of three independent replicates.

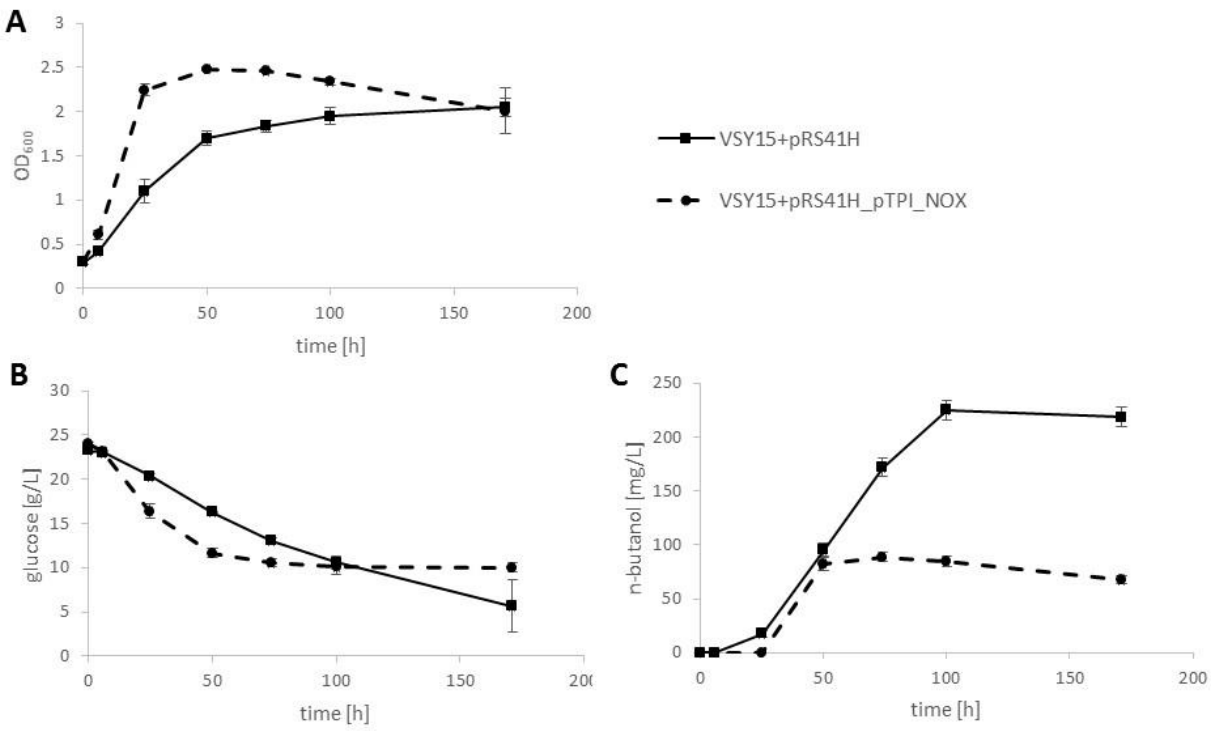

Figure S7: Aerobic fermentation of VSY15 containing NADH oxidase nox from *Streptococcus pneumoniae.*

Shown are values of OD_{600} (A), glucose (B) and n-butanol (C) concentration of fermentations in SMD media with VSY15 (Δ*adh1-6 sfa1 gpd2*, n-butanol pathway genes, *coaA* and *adhE*$^{A267T/E568K/R577S}$, pADH1_*FMS1*) with pRS41H_pTPI_NOX (dashed line) or empty vector pRS41H as a control (solid line).

Table S1: Relevant primers for this study

The abbreviations within the primer names were used as follows: forward primer (fw), reverse primer (rev), overlap (ov). Other primers for construction of all strains and plasmids are available in [2].

Primer name	Sequence 5'-3'	Description
vsp329_pVS4-Kass_ovtACS2_rev	ATTACGAAATTTTTCTCATTTAAGGAAAAATATAAG GATGAGAAAGTGAAATCGG	fusion-PCR for *adhE* $^{A267T/E568K/R577S}$ in *ACS2*
vsp330_pVS4-Kass_ovpACS2_fw	AGAATACAGGAAAGTAAATCAATACAATAATAAAA TCAGGCCTAGGCGACAACCC	fusion-PCR for *adhE* $^{A267T/E568K/R577S}$ in *ACS2*
vsp331_pACS2_ovpVS4-Kass_rev	GGGTTGTCGCCTAGGCCTGATTTTATTATTGTATTG ATTTACTTTCC	fusion-PCR for *adhE* $^{A267T/E568K/R577S}$ in *ACS2*
vsp332_tACS2_ovpVS4-Kass_fw	TCACTTTCTCATCCTTATATTTTTCCTTAAATGAGAA AAATTTCGTAATG	fusion-PCR for *adhE* $^{A267T/E568K/R577S}$ in *ACS2*
vsp333_tACS2_rev	AACAAGGCAAAATAGCGTTAACAACC	verification chr. integration of *adhE*
vsp334_pACS2_fw	TTTCCTGTGAGAAGTTTAAATCCACTAAGG	verification chr. integration of *adhE*
vsp335_ACS1_rev	GGTGCGGCAAATGGCCCG	test-PCR within gene (A6) for *ACS1*
vsp336_ACS1_fw	GGGCGTTCGCAAGGGCG	test-PCR within gene (A5) for *ACS1*
vsp337_ACS2_rev	GGCAGGTACAACTTCTGGGTTGGCC	test-PCR within gene (A6) for *ACS2*
vsp338_ACS2_fw	TTGGCTGTGGCTCGTATTGGTGC	test-PCR within gene (A5) for *ACS2*
vsp339_SFA1_rev	CACCAGCGGCAGCCACACC	test-PCR within *SFA1*
vsp340_SFA1_fw	GGGCCACGAAGGAGCCGG	test-PCR within *SFA1*
vsp368_CC-ACS1_fw	CCAAGACGTGTCTCACGCCTGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGG	CrisprCas for *ACS1* deletion
vsp368_CC-ACS1_rev	AGGCGTGAGACACGTCTTGGGATCATTTATCTTTCA CTGCGGAG	CrisprCas for *ACS1* deletion
vsp370_CC-ACS1-Donor	TCCTCCCAGAAAAACAATCTGTTTATTACCCGACAT TCTTCACTTTATGGGCAGTCCTTGTATCGACTTGCTC ATTATAA	CrisprCas for *ACS1* deletion
vsp371_pFMS1_ovtHis3_rev	ATTTTTATAGGTTAATGTCATGCTTATATACAATATA ATAATATGTAGGTAAACGTG	fusion-PCR for pADH1-*FMS1*
vsp372_pFMS1_fw	AATAGAAATAAGTTTGAGCAGTTCAAG	fusion-PCR for pADH1-*FMS1*
vsp373_tHis3_ovtADH1_rev	CCCCCTTCTCTACTAGCATTGGACTTAATGAGCTGA TTTAACAAAAATTTAACGC	fusion-PCR for pADH1-*FMS1*
vsp374_pHis3_ovpFMS1_fw	ACCTACATATTATTATATTGTATATAAGCATGACATT AACCTATAAAAATAGGCG	fusion-PCR for pADH1-*FMS1*
vsp375_pADH1_ovFMS1_rev	TTTGGCTGGTGAAACTGTATTCATTGTATATGAGAT AGTTGATTGTATGC	fusion-PCR for pADH1-*FMS1*
vsp376_pADH1_ovtHis3_fw	AAATTTTTGTTAAATCAGCTCATTAAGTCCAATGCT AGTAGAGAAGG	fusion-PCR for pADH1-*FMS1*
vsp377_FMS1_rev	CTAAGTGTTGATGGAATTCTAACTCTG	fusion-PCR for pADH1-*FMS1*
vsp378_FMS1_ovpADH1_fw	GCATACAATCAACTATCTCATATACAATGAATACAG TTTCACCAGCC	fusion-PCR for pADH1-*FMS1*
vsp379_FMS1_rev	GGCCTCATTCCGTCGATGACATCC	verification fusion-PCR for pADH1-*FMS1*
vsp380_pFMS1_fw	CTGCACGCAGGGATTGCCG	verification fusion-PCR for pADH1-*FMS1*
vsp388_CC-ALD6_fw	AAAACTTTGGCCTTAGCCCGGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGG	CrisprCas for deletion of *ALD6*
vsp389_CC-ALD6_rev	CGGGCTAAGGCCAAAGTTTTGATCATTTATCTTTCA CTGCGGAG	CrisprCas for deletion of *ALD6*
vsp390_CC-ALD6-Donor	AACATCTTTAACATACACAAACACATACTATCAGAA TACATGTACCAACCTGCATTTCTTTCCGTCATATACA CAAAATA	CrisprCas for deletion of *ALD6*

vsp391_tALD6_rev	GCGAAATGGCAGTACTCGGGGG	verification *ALD6*-deletion
vsp392_pALD6_fw	TCGTAATAAATTCGGGGTGAGGGGG	verification *ALD6*-deletion
vsp393_ALD6_rev	TACCGGCCTTCAACATCTTGGCC	test-PCR within gene (A5) for *ALD6*
vsp394_ALD6_fw	TCCACGACACTGAATGGGCTACCC	test-PCR within gene (A6) for *ALD6*
vsp398_ERG10_ovpHXT7_fw	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCA AAAAATGTCTCAAAACGTTTACATTG	bottleneck-analysis
vsp399_ERG10_ovtFBA1_rev	AATACTCATTAAAAAACTATATCAATTAATTTGAATT AACTTAAATCTTTTCAATGACAATAGAGG	bottleneck-analysis
vsp400_hbd_ovpHXT7_fw	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCA AAAAATGAAGAAGGTTTGTGTTATTGG	bottleneck-analysis
vsp401_hbd_ovtFBA1_rev	AATACTCATTAAAAAACTATATCAATTAATTTGAATT AACTTACTTAGAGTAATCGTAGAAACCC	bottleneck-analysis
vsp402_crt_ovpHXT7_fw	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCA AAAAATGGAATTGAACAACGTCATC	bottleneck-analysis
vsp403_crt_ovtFBA1_rev	AATACTCATTAAAAAACTATATCAATTAATTTGAATT AACTTATCTGTTCTTGAAACCTTCAATC	bottleneck-analysis
vsp404_tdTer_ovpHXT7_fw	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCA AAAAATGATTGTTAAGCCAATGGTTAGAAACAAC	bottleneck-analysis
vsp405_tdTer_ovtFBA1_rev	AATACTCATTAAAAAACTATATCAATTAATTTGAATT AACTTAAATTCTGTCGAATCTTTCAACTTCAGC	bottleneck-analysis
vsp406_adhE2_ovpHXT7_fw	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCA AAAAATGAAGGTTACCAACCAAAAG	bottleneck-analysis
vsp407_adhE2_ovtFBA1_rev	AATACTCATTAAAAAACTATATCAATTAATTTGAATT AACTTAGAAAGACTTAATGTAGATATCCTTC	bottleneck-analysis
vsp408_eutE_ovpHXT7_fw	AAACACAAAAACAAAAAGTTTTTTTAATTTTAATCA AAAAATGAACCAACAAGATATTGAACAAGTTG	bottleneck-analysis
vsp409_eutE_ovtFBA1_rev	AATACTCATTAAAAAACTATATCAATTAATTTGAATT AACTTAAACAATTCTGAAAGCATCGACC	bottleneck-analysis

Table S2: Yeast promoters and terminators used for the expression of n-butanol pathway genes and endogenous pantothenate synthesis in this study. DNA sequences were amplified from chromosomal DNA of CEN.PK2-1C. When not otherwise indicated, promoters were taken from 1-500 bps before and terminators 1-300 bps behind the respective open reading frames. Either all fragments were integrated into the chromosome via an integration cassette or introduced via vectors (in case of ^{Sc}*ALD2* and ^{Sc}*PAN6*), except for *FMS1*, whose promoter was exchanged in the genome. Genes from *Saccharomyces cerevisiae* (Sc), *Clostridium acetobutylicum* (Ca), *E.coli* (Ec), *Treponema denticola* (Td) are indicated by prefixes in superscript.

promoter	gene	terminator
*pHXT7**	^{Sc}*ERG10*	*tVMA16*
pPGK1	^{Ca}*hbd*	*tEFM1*
pTPI1	^{Ca}*crt*	*tYHI9*
pPYK1	^{Td}*ter*	*tIDP1*
*pADH1***	^{Ca}*adhE2*	*tRPL3*
pTDH3	^{Ec}*eutE*	*tRPL41B*
pPFK1	^{Ec}*adhE* $^{A267T/E568K/R577S}$	*tDIT1*
*pPMA1****	^{Ec}*coaA*	*tVMA2*
*pADH1***	^{Sc}*FMS1*	*tFMS1*
pFBA1	^{Sc}*ALD2*	*tPRC1*
*pGPM1*****	^{Sc}*PAN6*	*tNAT5*

* (-1 to -389 bp; [3])
** (-1 to -800 bp; [4])
*** (-1 to-575 bp, -700 to -925 bp; [5])
**** (-1 to -395 bp; [6])

Table S3: Statistical analysis of n-butanol production

A t-test was performed (unpaired, two-tailed, confidence: 95%) with program GraphPad Prism 5. n-Butanol titers of strains without or with additional adhE triple mutant (pVS4ev and VSY13 with genomic integration of *adhE*$^{A267T/E568K/R577S}$) of table 2 were compared (strain 1 with strain 2).

Strain 1	Strain 2	p-value of titer
VSY10	VSY13	0.24
VSY15	VSY15 + pVS4ev	0.27
VSY19	VSY19 + pVS4ev	0.57

References

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3. Becker J, Boles E. A modified *Saccharomyces cerevisiae* strain that consumes L-arabinose and produces ethanol. Appl Environ Microbiol. 2003;69:4144–4150.
4. Tornow J, Santangelo GM. Efficient expression of the *Saccharomyces cerevisiae* glycolytic gene *ADH1* is dependent upon a cis -acting regulatory element (UASRPG) found initially in genes encoding ribosomal proteins. Gene. 1990;90:79–85.
5. Capieaux E, Vignais ML, Sentenac A, Goffeau A. The yeast H+-ATPase gene is controlled by the promoter binding factor TUF. J Biol Chem 1989;264:7437-7446.
6. Rodicio R, Heinisch JJ, Hollenberg CP. Transcriptional control of yeast phosphoglycerate mutase-encoding gene. Gene. 1993;125:125–133.