Electronic Supplementary Material (ESI) for RSC Advances.


Supporting Information

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## 1. Materials and methods

### Cloning, expression and purification

*Acetobacter pasteurianus* pyruvate decarboxylase (*Ap*PDC), *Zymomonas mobilis* PDC (*Zm*PDC), *Zymobacter palmae* PDC (*Zp*PDC), PDC inferred from ASR (PDB: 5NPU) and Rosetta-based *Zm*PDC mutant (PDB: 5TMA) are cloned into pET24 using *Nde*I and *Xho*I bearing a C-terminal hexahistidine tag. Two, new PDCs from *Candida glabrata* (*Cg*) and *Zygosaccharmoyces rouxii* (*Zr*) are cloned into pET24 using the same procedures. All PDCs, except *Zm*PDC, are ordered codon-optimised for *Escherichia coli* and purchased from either GeneArt (Germany) or ATG Biosynthesis (Germany).

After cloning all PDCs into pET24, chemically competent *E. coli* BL21 (DE3) cells were transformed with plasmids encoding different PDCs, and the cells were plated on LB agar containing Kanamycin 50 µg/ml. The plates were incubated overnight at 37 °C. Pre-cultures were prepared by inoculating a single colony from the plates into 10-ml LB containing Kanamycin 50 µg/ml and shaken overnight at 150 rpm, 37 °C. On the following day, the 10 ml pre-cultures were transferred to 250 ml autoinduction media containing Kanamycin 100 µg/ml in 1 L baffled flasks.[1] The cultures were incubated at 37 °C, 120 rpm, for 3 h before the temperature was adjusted to room temperature (25 °C) for overnight protein expression. Protein purifications followed the same procedures as described previously, and 50 mM phosphate buffer, pH 6.5, was used as the final buffer.[2] Each purified protein was flash frozen in liquid nitrogen and stored at −80 °C before further characterisations.

### Kinetic characterisation

For kinetic characterisation, a standard coupled assay, with yeast alcohol dehydrogenase (Sigma), was used. The reaction solution contained different sodium pyruvate concentrations (0–50 mM), 50 mM phosphate buffer, pH 6.5, 1 U/ml yeast ADH (Sigma), 1 mM NADH, 0.1 mM thiamine diphosphate (TDP) and 0.5 mM $MgSO_4$. 180 µl of reaction solution was transferred to a 96-well, F-bottom plate (Greiner, Germany) that had been pre-filled with 20 µl of the enzyme solution to start the reaction. The reaction was monitored at 340 nm, 25 °C using Multiskan (Thermo-scientific). Specific enzyme activity (U/mg) was defined as the amount of enzyme required for the consumption of 1 µmol NADH per minute. Three technical replications were performed. The kinetic data are presented in Table 1.

### Kinetic stability

There are two parameters used to measure kinetic stability. The first one is $T_{50}^{1h}$, defined as the temperature at which 50% of initial activity remained after 1 h incubation. To determine the $T_{50}^{1h}$, 1 mg/ml of wild-type (WT) enzymes or variants containing 0.1 mM TDP and 5 mM $MgSO_4$ in 50 mM phosphate buffer, pH 6.5, were incubated in a 96 PCR plate at different temperatures for 1 h. After the heat challenge, dilution buffer (50-mM phosphate buffer, pH 6.5, 0.1 mM TDP and 5 mM $MgSO_4$) was added. Twenty microliters of the mixture was transferred to a new 96 MTP. 180 µl of substrate solution (50 mM phosphate buffer, pH 6.5, 0.1 mM TDP, 5 mM $MgSO_4$ and 25 mM sodium pyruvate) was added to determine residual activity by direct measurement of pyruvate consumption at 315 nm, at 25°C. One hundred percent activity is defined as the activity of the enzyme after incubation at 25°C for 1 h. Three technical replications were conducted. The $T_{50}^{1h}$ data are presented in Table 1.

The second parameter is the half-life of the enzyme ($t_{1/2}$). For this parameter, 1 mg/ml of WT *Ap*PDC and Variant 2, containing 0.1 mM TDP and 5 mM $MgSO_4$ in 50 mM phosphate buffer, pH 6.5, were transferred to several 8-strip, PCR cups. The PCR cups were incubated at 65, 70 and 75 °C. At different,

each PCR cup was removed from the PCR, and dilution buffer was added. Residual activity was measured by direct assay at 315 nm. Half-life is defined as the time at which the enzyme retained 50% of its initial activity. The temperature-dependent, deactivation data are presented in Figure 1.

**Thermodynamic stability by Thermofluor**

Melting temperatures ($T_m$) were measured, as described previously.[2] $T_m$ data are presented in Table 1

**Stability in the presence of butanol**

There are two parameters evaluated for the stability of WT *Ap*PDC and Variant 2. The first is stability in the presence of 9 vol% butanol at 50 °C. At this concentration and temperature, butanol will start to form two phases with water. Both enzymes were diluted (end concentration 1 mg/ml) in 50 mM phosphate buffer, pH 6.5, containing 9 vol% butanol, 0.1 mM TDP and 5 mM $MgSO_4$). The solutions were then aliquoted 20 µl in 8-strip, PCR cups and incubated in a PCR machine. At different intervals, each PCR cup was removed from the PCR, and dilution buffer was added. Residual activity was measured by direct assay at 315 nm, as described above. Half-life is defined as the time at which the enzyme retained 50% of its initial activity. Three independent technical replications were performed. The butanol and temperature-dependent deactivations are presented in Figure 2A.

In the second parameter, different concentrations of butanol (0–9 vol%) were added to the enzyme solution (end concentration 1 mg/ml) in 50 mM phosphate buffer, pH 6.5, 0.1 mM TDP and 5 mM $MgSO_4$. The 20 µl aliquots were transferred to a 96 PCR plate (Brand, Germany) and incubated at 60 °C for 1h. After incubation, dilution buffer was added, and 20 µl of the solution was transferred to a 96 MTP. The remaining activity was measured by direct assay after the addition of 180 µl substrate solution, as described above. Three independent technical replications were performed. The butanol stabilities of WT *Ap*PDC and Variant 2 are presented in Figure 2B.

**Stability in the presence of ethanol**

The phosphate buffer of WT PDCs or variants was exchanged for 50 mM MOPS, pH 6.5, using PD-10 desalting column (GE Healthcare, Germany) prior to the stability experiments because phosphate buffer precipitates in the presence of high ethanol concentrations. Different volumes of anhydrous ethanol were added to each enzyme solution, in the presence of 0.1 mM TDP and 5 mM $MgSO_4$, to make a final enzyme concentration of 1 mg/mL with ethanol concentrations ranging from 0–50 vol%. Twenty microliters of the mixture was transferred to a 96 PCR plate and incubated at 50°C for 1 h. After incubation, dilution buffer (50 mM MOPS, pH 6.5, 0.1 mM TDP and 5 mM $MgSO_4$) was added to the PCR plate, and 20 µl of the solution was transferred to a 96 MTP. The remaining activity was measured by direct assay after the addition of 180 µl substrate solution (50 mM MOPS, pH 6.5, 0.1 mM TDP, 5 mM $MgSO_4$ and 25 mM sodium pyruvate).

**Library development (StEP)**

The three most stable PDC, *Ap*PDC, *Zm*PDC and *Zp*PDC, were used as the parental templates for the staggered extension process (StEP). Prior to the StEP, all three PDCs were re-cloned into a modified pBAD plasmid with *Xba*I and *Xho*I. Standard StEP protocol was used, with slight modification.[3] In brief, 10 ng/µl of each plasmid was mixed with dNTPs 2 mM, GoTaq polymerase 1 U, GoTaq green buffer 1x, forward primer 15 mM, reverse primer 15 mM and double-distilled water, to make a 100 µl PCR mix. The mixture was divided into 8 x 12.5 µl aliquots and transferred to 8-strip, PCR cups. The PCR

programme used was as follows: 5 min initial denaturation at 95 °C, 30 s denaturation at 94 °C, 7 s annealing at 50 °C, 5 s elongation at 72 °C and repetition of the denaturation step to elongation step 130 times before holding indefinitely at 16 °C. The PCR products were then separated using 1% agarose gel (Serva, Germany). A strong band, with a size of about 1800 bp, was observed. The DNA band was excised and purified using a gel purification kit (Macherey Nagel). Purified DNA was digested with *Xba*I and *Xho*I and cloned into pET24. *E. coli* BL21 (DE3) cells were transformed, with the pET24 harbouring the library and plated on LB agar containing Kanamycin 50 µg/ml. The plates were incubated overnight at 37°C.

**High-throughput screening**

Single colonies from the library plates were inoculated into 96 deep-well plates (DWP) containing 800 µl autoinduction media with Kanamycin 100 µg/ml. *E. coli* BL21 (DE3) cells harbouring *Ap*PDC were used as a positive control and inoculated randomly into 8 wells in each of the 96 DWPs, because *Ap*PDC has the highest thermostability compared with *Zm*PDC and *Zp*PDC (Table 1). The 96 DWPs were shaken in a plate shaker at 1000 rpm, 30°C, and incubated overnight. On the following day, 50 µl of the culture was transferred to a 384 MTP (Brand, Germany) that had been pre-filled with glycerol 50% using a Tecan, liquid-handling station for backup. The plates were kept at −80°C. Another 200 µl of the culture was transferred to a 96 U-bottom plate (Sarstedt, Germany), and the plate was centrifuged at 4000 xg for 15 min to remove the supernatant. Two hundred microliters of lysis solution (50 mM phosphate buffer, pH 6.5, 0.1 mM TDP, 5 mM $MgSO_4$, 1 mg/ml lysozyme, 0.5 mg/ml Polymyxin B sulfate and 10 µg/ml DNAse) was added, and the plates were shaken at 1000 rpm, 37°C for 1 h. After the lysis step, a heat challenge was performed by incubating the plates in a water bath, at 75°C, for 30 min. Several colonies showing residual activity higher than three times the standard deviation, were subjected to a second screening. About 800 colonies were selected in the first screening.

In the second screening, the colonies that appeared to have higher stability in the first screening were inoculated into LB liquid containing Kanamycin 50 µg/ml from the backup plates for plasmid purification. *E. coli* Bl21 (DE3) cells were transformed with the plasmids and grown on LB agar containing 50 µg/ml Kanamycin. Eight or more colonies were selected from each variant, and all the screening steps described above were repeated. Plasmid from the variants that retained stability was sent for sequencing (Eurofins, Germany).

**Site saturation mutagenesis with QuikChange**

For QuikChange, the same protocol, as described previously, was followed.[2] pET24a containing gene encoding Variant 1 was used for the templates. The Primers used are listed in the Section 7 of Supporting Information. The same first and second screening procedures described above were employed. *E. coli* BL21 (DE3) cells harbouring Variant 1 plasmid were used as a positive control, and the heat challenge was undertaken at 80°C for 30 min in the water bath.

## 2. Kinetic and thermodynamic stability

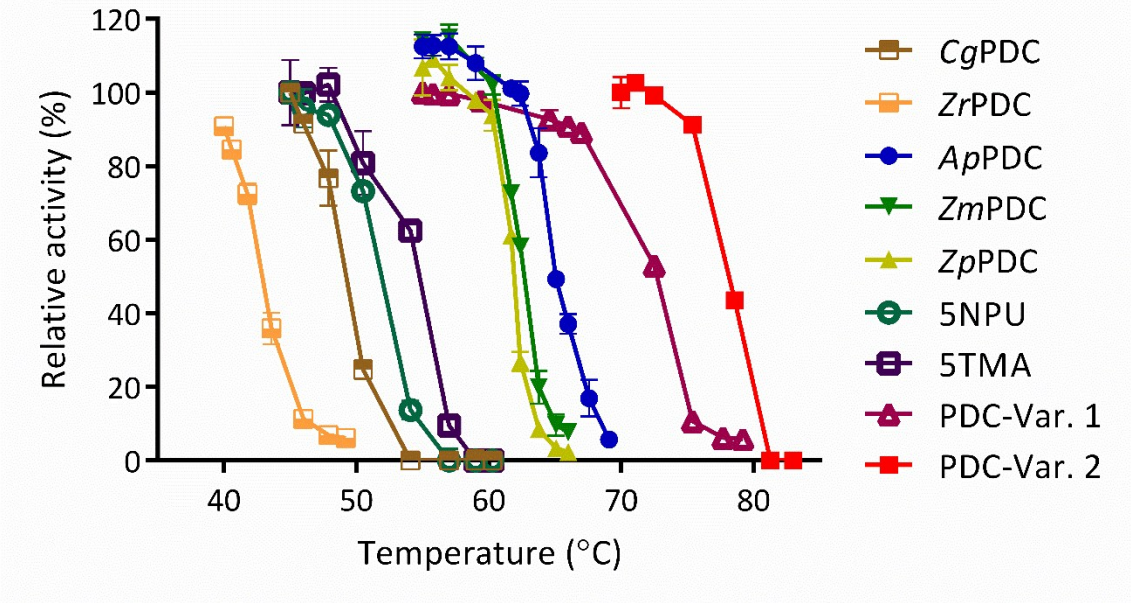


**Figure S1A.** Stability of WT PDCs and variants after 1 h incubation at different temperatures. 100% activity is defined as the activity after 1 h incubation at 25 °C. *Cg* is *Candida glabrata*, *Zr* is *Zygosaccharomyces rouxii*, *Ap* is *Acetobacter pasteurianus*, *Zm* is *Zymomonas mobilis* and *Zp* is *Zymobacter palmae*. 5NPU was obtained by ancestral sequence reconstruction.[4] 5TMA is derived from *Zm*PDC by Rosetta design.[5]

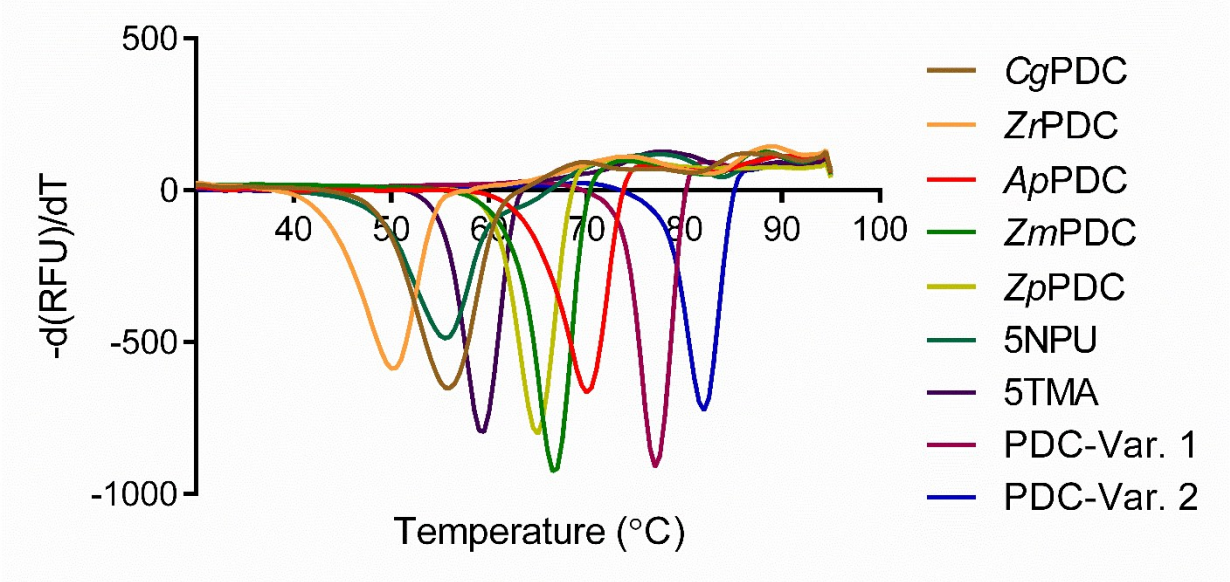


**Figure S1B.** First-derivative curve of $T_m$ of PDCs measured by Thermofluor assay. *Cg* is *Candida glabrata*, *Zr* is *Zygosaccharomyces rouxii*, *Ap* is *Acetobacter pasteurianus*, *Zm* is *Zymomonas mobilis* and *Zp* is *Zymobacter palmae*. 5NPU was obtained by ancestral sequence reconstruction.[4] 5TMA is derived from *Zm*PDC by Rosetta design.[5]

3. Crossover of bacterial PDCs

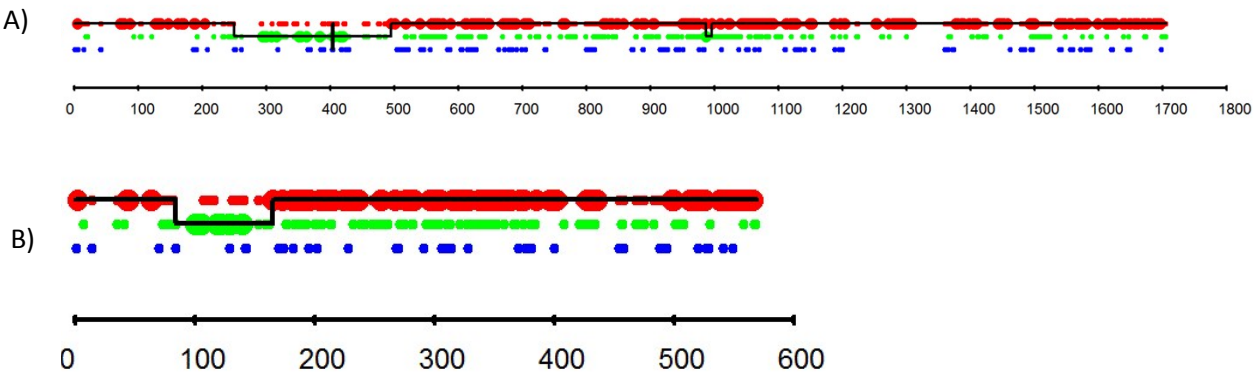


**Figure S2.** Crossover of Variant 1 in comparison with its parental PDC at nucleotide level (A) and amino acid level (B). Red is *Ap*PDC, green is *Zp*PDC and blue is *Zm*PDC. Large dots represent 100% parental match and small dots represent more than one parental match.

Crossover was calculated using Xover 3.0.[6] The results suggested that crossover on the nucleotide level happened at least six times, while on the amino acid level, it happened only twice. This result indicated that some mutations are silent. From the amino acid level, Variant 1 showed major changes in comparison with *Ap*PDC, starting from amino acid at position 83 up to 143.

4. Comparison between wild-type pyruvate decarboxylase from *Acetobacter pasteurianus* and thermostable variants

Amino acid sequence of Variant 2. The substitutions, in comparison with WT *Ap*PDC, are marked yellow.

MTYTVGMYLAERLVQIGLKHHFAVAGDYNLVLLDQLLLNKDMKQIYCCNELNCGFSAEGYARSNGAAAAVVTFSV
GAISAMNAIGGAYAENLPVILISGSPNTNDYGTGHILHHTIGTTDYNYQLEMVKHVTCAAESIVSAEEAPAKIDH
VIRTALRERKPAYLDIACNIASEPCVRPGPVSSLLSEPEIDHTSLKAAVDATVALLEKSASPVMLLGSKLRAANA
LAATETLADKLQCAVTIMAAAKGFFPEDHAGFRGLYWGEVSNPGVQELVETSDALLCIAPVFNDYSTVGWSAWPK
GPNVILAEPDRVTVDGRAYDGFTLRAFLQALAEKAPARPASAQKSSVPTCSLTATSDEAGLTNDEIVRHINALLT
SNTTLVAETGDSWFNAMRMTLPRGARVELEMQWGHIGWSVPSAFGNAMGSQDRQHVVMVGDGSFQVTAQEVAQMV
RYELPVIIFLINNRGYVIEIAIHDGPYNYIKNWDYAGLMEVFNAGEGHGLGLKATTPKELTEAIARAKANTRGPT
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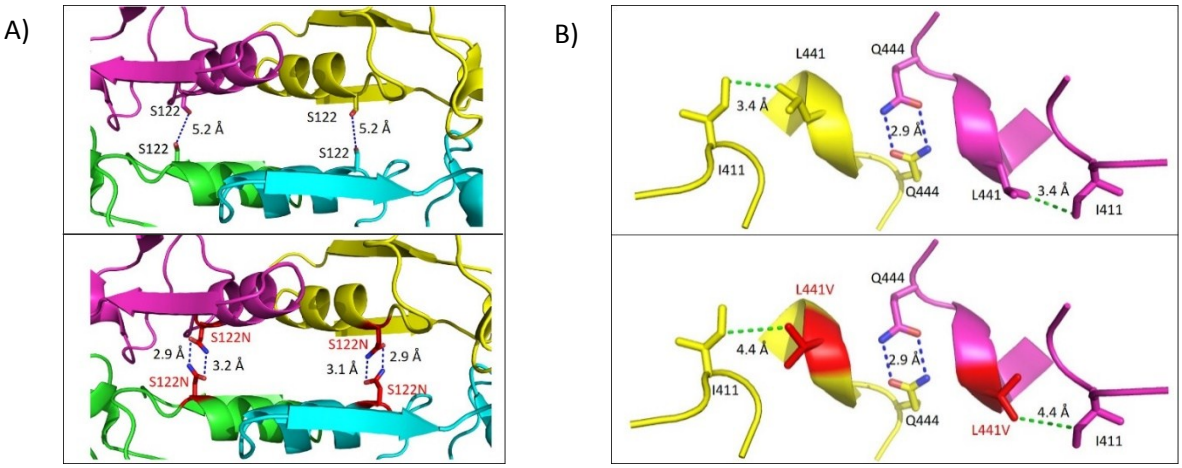


**Figure S3** Amino acid substitutions in Variant 2 that led to improved thermostability. The illustration was obtained from the crystal structure of *Ap*PDC (PDB:2vbi). The amino acid substitutions are coloured in red, while the original amino acids are coloured according to the backbone. 2vbi is depicted as a homotetramer and consists of two homodimers (purple-yellow and green-cyan). (**A)** Substitution from serine, at position 122, to asparagine may provide four, new electrostatic interactions between two dimers in PDC tetrameric form. (**B)** Change from leucine to valine, at position 441, may decrease its previous hydrophobic interaction to I411, leading to better electrostatic interaction between Q444 from each monomer.

Variant 1 obtained from StEP contained 13 point substitutions. Until the crystal structure is elucidated, the explanation for the individual substitutions may not be straightforward. However, one notable change is S122N, presented in Figure S3.

5. Ethanol stability

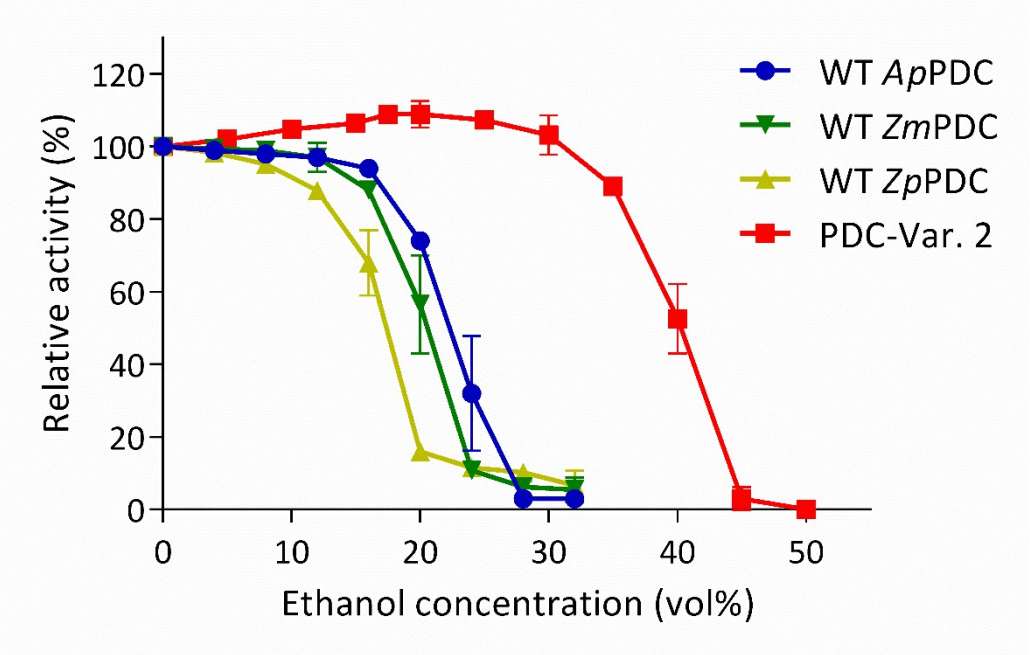


**Figure S4.** Stability of different PDC variants in the presence of ethanol, at 50°C, after 1 h incubation. 100% activity is defined as the activity after 1 h incubation, at 50 °C, of the enzymes at 0 vol% ethanol. *Ap* is *Acetobacter pasteurianus*, *Zm* is *Zymomonas mobilis* and *Zp* is *Zymobacter palmae*.

6. Butanol stability

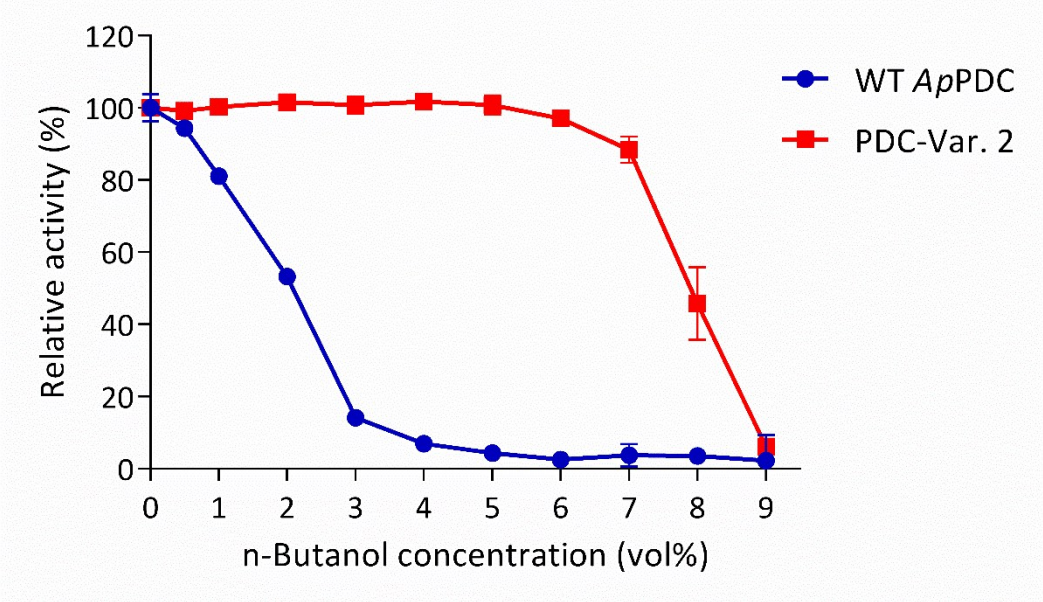


**Figure S5.** Stability of different WT *Ap*PDC variants in the presence of butanol, at 60°C, after 1 h incubation. 100% activity is defined as the activity after 1 h incubation, at 60 °C, of the enzymes at 0 vol% n-butanol. *Ap* is *Acetobacter pasteurianus.*

7. Primer used in this study

Table S6. List of the primers used. Mutations are written in italics.

| Primer | Sequence (5´→ 3´) |
|---|---|
| pBAD_Fwd | ATGCCATAGCATTTTTATCC |
| pBAD_Rev | CCTTGAATACACCATGTAGTG |
| pBADQC_XbaI.F | TACCCG*TCTAGA*GGGCTAACAGGAGGAATTAACC |
| pBADQC_XbaI.R | TTAGCCC*TCTAGA*CGGGTATGGAGAAACAGTAGAG |
| PDC-Var.1_D120NNS.F | ACCACC*NNS*TACAATTATCAGCTGGAAATGGTGAAAC |
| PDC-Var.1_D120NNS.R | AATTGTA*SNN*GGTGGTGCCAATGGTATGATGCAGAATATG |
| PDC-Var.1_G256NNS.F | ATCATGCA*NNS*TTTCGTGGTCTGTATTGGGGTG |
| PDC-Var.1_G256NNS.R | ACGAAA*SNN*TGCATGATCTTCCGGAAAAAAACCTTTTGC |
| PDC-Var.1_G294NNS.F | ACCGTT*NNS*TGGAGCGCATGGCCGAAAGGTC |
| PDC-Var.1_G294NNS.R | GCTCCA*SNN*AACGGTGCTATAATCATTAAAAACCGG |
| PDC-Var.1_D310NNS.F | GAACCG*NNS*CGTGTTACCGTTGATGGTCGTG |
| PDC-Var.1_D310NNS.R | AACACG*SNN*CGGTTCGGCCAGAATAACATTCG |
| PDC-Var.1_D320NNS.F | GCATAC*NNS*GGTTTTACCCTGCGTGCCTTTCTG |
| PDC-Var.1_D320NNS.R | AAAACC*SNN*GTATGCACGACCATCAACGGTAACACG |
| PDC-Var.1_T395NNS.F | CGTATG*NNS*CTGCCTCGTGGTGCACGTGTTG |
| PDC-Var.1_T395NNS.R | AGGCAG*SNN*CATACGCATTGCATTAAACCAGCTATC |
| PDC-Var.1_L441NNS.F | TTTCAG*NNS*ACCGCACAAGAGGTTGCACAGATG |
| PDC-Var.1_L441NNS.R | TGCGGT*SNN*CTGAAAGCTACCATCACCAACCATAAC |
| PDC-Var.1_I465NNS.F | TTACGTG*NNS*GAAATTGCCATTCATGATGGTCCGTAC |
| PDC-Var.1_I465NNS.R | AATTTC*SNN*CACGTAACCGCGATTGTTAATCAGGAAG |

## 8. References


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