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To beat the heat – engineering of the most thermostable pyruvate decarboxylase to date†

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Pyruvate decarboxylase (PDC) is a key enzyme for the production of ethanol at high temperatures and for cell-free butanol synthesis. Thermostable, organic solvent stable PDC was evolved from bacterial PDCs. The new variant shows >1500-fold-improved half-life at 75 °C and >5000-fold-increased half-life in the

For many years, bioethanol has been used widely as an additive in gasoline or as a fuel by itself.¹ To avoid competition with food, production of ethanol from plant-derived starch or sugar (first generation) has shifted to lignocellulosic biomass (second generation). To date, most of the bioethanol produced industrially still utilises yeasts as the main fermentation organism.¹⁻³ However, in recent years, several efforts have been made to use thermophilic microorganisms as the new framework.^{1,3} The main motivating forces behind this are the volatility of ethanol at higher temperatures, which facilitates easy product removal, reduced risk of contamination and feasibility of consolidated bioprocessing (CBP). In this process, cellulolytic ethanologenic thermophilic microorganisms are applied, thus reducing the energy consumption required for heat exchange between biomass pre-treatment, fermentation and product separation and to avoid cellulase inhibition by accumulating glucose.3,4

presence of 9 vol% butanol at 50 °C.

There are two major pathways for producing ethanol from pyruvate, the central metabolite in glycolysis: non-oxidative decarboxylation via pyruvate decarboxylase (PDC) and oxidative decarboxylation via pyruvate dehydrogenase (PDH) or pyruvate ferredoxin oxidoreductase (PFOR). Non-oxidative ethanol productions are commonly found in yeasts and other ethanologenic bacteria, while PDH and PFOR routes are described in thermophilic microorganisms.^{3,4} PDC mode may offer advantages compared with PDH and PFOR, as it is thermodynamically more favourable and will not lead to any other

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organic acid by-products.3-5 In fact, all industrially relevant ethanol producing organisms use PDC. Thermostable PDCs are, therefore, suggested to play an important role in creating homoethanologenic, thermophilic microorganisms.5,6

Until recently, there were only limited efforts described in literature for improving the thermostability of PDC. Ancestral sequence reconstruction, supposedly a powerful method of resurrecting ancient thermostable enzymes, did not yield more thermostable PDC.7 A computational Rosetta-based design resulted in several variants with improved stability, determined by differential interference contrast (DIC) microscopy; unfortunately, the work was not corroborated by kinetic activity and stability data.8 Another effort to redirect the specificity of a thermostable, acetolactate synthase to perform a PDC-like reaction by conventional directed evolution did not yield variants either, with activity similar to that of bacterial PDC.9 Thus, creating thermostable PDC variants remains as the main challenge towards second generation ethanol production by thermophilic microorganisms.

Besides acting as an important enzyme for ethanol production, PDC has also been applied in the in vitro production of nbutanol (Scheme 1).10,11 Butanol together with other longerchain alcohols, such as 1-propanol, isobutanol and



Scheme 1 Simplified pathway to produce ethanol and 1-butanol from lignocellulosic biomass via pyruvate decarboxylase (PDC). There are two major pathways for producing 1-butanol via pyruvate: CoAdependent or proline-dependent condensation. ADH is an alcohol dehydrogenase

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isopentanol, is regarded as the next-generation biofuel, due to its closer resemblance to traditional gasoline.¹² Thus, there is an increasing demand to produce longer-chain alcohols sustainably.¹² Industrially, butanol is still produced from petroleumderived propene utilizing Co-catalyst, H₂, and CO *via* oxo synthesis and Reppe process.¹³ Albeit its efficiency, production of butanol from petroleum is not sustainable.

In a more sustainable way, butanol can be produced via an acetone-butanol-ethanol (ABE) process, utilising Clostridium acetobutylicum.12,14 Recent work on metabolic engineering has demonstrated the possibility of producing butanol in other microorganisms.^{15,16} However, one of the major challenges in butanol fermentation is still the toxicity of butanol to many microorganisms at relatively low concentrations (<2 vol%).17,18 This effect, however, is less pronounced for the enzymes catalysing butanol production in vitro.10,19 Many enzymes can already tolerate higher organic solvent concentrations than the corresponding microorganisms.20 Furthermore, engineering enzyme stability towards organic solvents is less challenging than evolving microorganisms to withstand the same concentration of organic solvents.17,21 Therefore, in vitro butanol production emerges as a very promising alternative to traditional butanol fermentation. Thus, in this work, we focused on the engineering of PDC towards higher thermal stability for prospective ethanol production in thermophilic microorganisms and improved stability in butanol for applications in in vitro, artificial butanol synthesis.

Prior to engineering a PDC, we characterised two new yeast PDCs from *Candida glabrata* (*Cg*PDC) and *Zygosaccharomyces rouxii* (*Zr*PDC), as well as other PDCs reported in the literature, to determine the most suitable template. As presented in Table 1, the two new PDCs from the yeast are not thermostable and showed typical substrate cooperativity, as described for other yeast PDCs.²² Characterisation of bacterial PDCs showed similar findings to the previous report, with PDC from *Acetobacter*

 Table 1
 Summary of kinetic characterisations and thermostability of

 WT PDCs and variants
 Variants

Enzyme	$V_{\rm max} \left({\rm U \ mg^{-1}} \right)$	$K_{\rm m}$ (mM)	$T_{50}^{1 ha} (^{\circ}C)$	$T_{\rm m}^{\ b} (^{\circ} {\rm C})$
<i>Cg</i> PDC	22.2 ± 0.4	$9.1 \pm 0.2 \text{ h}: 2.02^{\circ}$	49.6	55.5
ZrPDC	16.7 ± 0.4	11.6 \pm 0.4 h: 2.39 c	42.9	50.5
ApPDC	80.3 ± 0.6	1.9 ± 0.1	64.9	70.0
ZmPDC	92.5 ± 0.7	1.3 ± 0.1	62.4	66.5
<i>Zp</i> PDC	$\textbf{79.7} \pm \textbf{0.4}$	0.8 ± 0.1	61.2	65.0
5NPU ⁷	20.7 ± 0.8	4.8 ± 0.4 h: 1.28^{c}	51.9	55.5
5TMA ⁸	87.0 ± 0.7	1.8 ± 0.1	54.7	59.5
PDC-Var. 1	91.7 ± 0.7	2.1 ± 0.1	73.1	77.0
PDC-Var. 2	71.6 ± 0.7	1.5 ± 0.1	78.5	82.0

^{*a*} $T_{50}^{1 \text{ h}}$ is defined as the temperature at which the enzyme gives 50% remaining activity after 1 h incubation (Fig. S1).²³ ^{*b*} Melting temperature was determined using Thermofluor assay (Fig. S1). ^{*c*} The Hill coefficient was determined according to the equation: $V = \frac{V_{\text{max}}[S]^{\text{h}}}{V_{\text{max}}[S]^{\text{h}}} Ca \text{ is Candida glabrata Tr is Twasaccharameters}$

 $V = \frac{r_{\max[I]}}{[K_m]^h + S^h}$. Cg is Candida glabrata, Zr is Zygosaccharomyces

pasteurianus (*ApPDC*) demonstrating the highest kinetic stability $(T_{50}^{1 \text{ h}})$ and melting temperature (T_{m}) .²² $T_{50}^{1 \text{ h}}$ is defined as the temperature at which 50% of the enzymes remained active after 1 h incubation, while T_{m} is defined as the temperature at which 50% of the enzymes are in an unfolded state.²³ In general, bacterial PDCs showed higher activity at 25 °C and overall stability $(T_{50}^{1 \text{ h}})$ and T_{m} than newly characterised *Cg*PDC and *Zr*PDC.

Another PDC that was reported to have improved stability, called 5TMA (Rosetta-designed ZmPDC), was also characterised in this study to fill the gap remaining due to the lack of data on activity and kinetic stability in the original study.8 It was claimed that 5TMA did not show any loss of molar ellipticity, measured by circular dichroism and that it was stable at 60 °C, observed by DIC microscopy. However, our kinetic stability studies based on activity suggested otherwise. We observed that 5TMA was less stable than wild-type (WT) ZmPDC, the parental PDC, in respect to $T_{50}^{1 \text{ h}}$ and T_{m} (Table 1). We could not explain this major discrepancy, except that we used a buffer of pH 6.5, while the previous work used pH 7.5.8 It is worth mentioning that during our $T_{50}^{1 h}$ experiment, we observed that 5TMA did not show any aggregation in contrast to other WT PDCs. This could explain why the authors did not observe a phase transition in DIC of 5TMA, but did for WT ZmPDC.

Our previous work on evolving thermostable, branchedchain α -keto acid decarboxylase from *Lactococcus lactis* (*LlK*dcA) showed that improved thermostability was also translated to increased stability against isobutanol.²⁴ The direct relationship between thermostability and organic solvent stability has also been described in other enzyme classes.^{21,25,26} Therefore, to save time and expense in finding PDC variants with increased thermostability and butanol-stability, we developed a simple screening platform to screen only for improved thermostability. For library development, staggered extensions process (StEP) was utilised.²⁷ StEP has been shown in previous work to be a robust and relatively easy approach for increasing thermostability.²⁷ Three PDCs from *Zymomonas mobilis* (*Zm*PDC), *Zymobacter palmae* (*Zp*PDC) and *Ap*PDC with relatively similar stability and activity, were chosen as parental templates.

After screening about 800 colonies, we obtained several variants that showed improved thermostability in comparison with our control (WT *ApPDC*). The most thermostable variant (renamed PDC-Var. 1) was selected, and the mutations were determined by DNA sequencing. PDC-Var. 1 showed the highest sequence similarity to *ApPDC* (98%), bearing 13 substitutions (Fig. S2 and S3†). In comparison with *ApPDC*, the most thermostable PDC reported to date, PDC-Var. 1 exhibited increases of 8.2 °C and 7.0 °C in $T_{50}^{1 \text{ h}}$ and T_{m} , respectively. Surprisingly, PDC-Var. 1 also showed improved activity in comparison with WT *ApPDC*, without significant change in K_{m} (Table 1). This indicated that increased thermostability is not always accompanied by a decrease in activity.²⁸ We later used this new variant as a new template to further increase its thermostability.

In a previous work with *Ll*KdcA, we successfully improved its thermal and isobutanol-stability by introducing seven amino acid substitutions (Var. 7M.D).²⁴ As *ApPDC* and *Ll*KdcA belong to the same α -keto acid decarboxylase family, we intended to

rouxii, Ap is Acetobacter pasteurianus, Zm is Zymomonas mobilis and Zp is Zymobacter palmae.

transfer the seven beneficial substitutions of Var. 7M.D to our new PDC-Var. 1. A similar screening procedure was applied. From the seven libraries, only one variant showed improved stability. We named the new variant PDC-Var. 2, which displays a change at position 441 of PDC-Var. 1 from isoleucine to valine (Fig. S3†).²⁴ PDC-Var. 2 exhibited $T_{50}^{1 \text{ h}}$ and T_{m} of 78.5 °C and 82.0 °C, respectively, meaning an improvement of 5.4 °C and 5.0 °C compared with PDC-Var. 1 or an increase of 13.6 °C and 12.0 °C compared with WT *Ap*PDC.

To check the stability of PDC-Var. 2 in comparison with WT ApPDC for ethanol fermentation applications via thermophilic microorganisms, both enzymes were incubated at 65 °C, 70 °C and 75 °C (Fig. 1). At 65 °C, WT ApPDC showed a first-order deactivation kinetics, with a half-life of 57 min, while PDC-Var. 2 showed a halflife of 18 h. This improvement represented an almost 19-fold increase. At higher temperatures, the stability effect of PDC-Var. 2 became even more apparent. At 70 °C, WT ApPDC completely lost its activity after 10 min of incubation, while PDC-Var. 2 retained 25% of initial activity after 24 h incubation. At 75 °C, WT ApPDC was completely deactivated after 2 min, while PDC-Var. 2 retained more than 20% of initial activity after 12 h incubation. Based on a firstorder kinetic deactivation, WT ApPDC exhibited half-lives of 1.2 min and 10.8 s at 70 °C and 75 °C, respectively. For PDC-Var. 2, the half-lives at 70 $^\circ$ C and 75 $^\circ$ C are 10.7 h and 7.3 h, respectively. As such, the improved stability of PDC-Var. 2 corresponded to a 522fold and a 2438-fold increased half-life at 70 °C and 75 °C (Fig. 1), respectively. PDC-Var. 2 also demonstrated improved stability towards ethanol by tolerating ethanol up to 40 vol% at 50 °C in comparison with WT ApPDC that showed stability up to only 22 vol% (Fig. S4[†]). We stress that this new improved characteristic is important for ethanol production in vitro.19

Biotechnological production of butanol is challenging, mainly due to its high toxicity for microbial cells. Until now, no microorganism has been found in nature that can grow at butanol concentrations higher than 4 vol%.²⁹ Recently, production schemes were proposed that utilise the solubility limit of butanol in water (9 vol% at 50 °C).^{10,11} Using solventstable enzymes and hands-on *in vitro* enzyme cascade, cellfree butanol production within a two-phase water/butanol system can be envisioned. Some enzymes from thermophilic microorganisms can already tolerate higher alcohol concentrations, such as aldolase from *Saccharolobus solfataricus* (previously known as *Sulfolobus solfataricus*), which was shown to retain full activity in a two-phase system.¹⁹

At 50 °C, the most thermostable WT PDC, *Ap*PDC, showed stability up to only 5.6 vol% butanol after incubation for 1 h (Fig. 2A). In 7 vol% butanol, WT *Ap*PDC was completely deactivated. As expected, PDC-Var. 2 demonstrated exceptional stability, by retaining more than 70% of its initial activity in 9 vol% butanol after 1 h incubation at 50 °C (Fig. 2A). Further kinetic stability studies revealed that WT *Ap*PDC was completely deactivated after 30 s incubation at 9 vol% butanol at 50 °C (Fig. 2B). PDC-Var. 2, however, maintained more than 40% of its initial activity after 24 h incubation and 15% of its initial activity after 48 h incubation (Fig. 2B). This corresponds to a half-life of 15 h and thus an over 5000-fold increase in half-life in comparison with WT *Ap*PDC.



Fig. 2 Stability of WT ApPDC and PDC-Var. 2 in the presence of butanol. (A) Stability of the WT ApPDC and the variant after incubation in different concentrations of butanol for 1 at 50 °C. Lines are drawn to ease reading. (B) Stability of the WT ApPDC and the variant over time in 9 vol% butanol at 50 °C. Standard deviations are shown from three independent technical replications.



Fig. 1 Kinetic stability of WT ApPDC in comparison with PDC-Var. 2 at 65 °C (A), 70 °C (B) and 75 °C (C). Thermal denaturations of the WT and PDC-Var. 2 followed first order kinetics at any given temperature. Half-lives of WT ApPDC are 57 min, 1.2 min and 10.8 s at 65, 70 and 75 °C, respectively. Half-lives of Variant 2 are 18, 10.7 and 7.3 h at 65, 70 and 75 °C, respectively. Standard deviations are shown from three independent technical replications.

When a higher temperature is desired, *e.g.* 60 °C, as indicated by previous work, WT *Ap*PDC could retain 50% of its activity only up to 2 vol% of butanol at 60 °C, while PDC-Var. 2 started to lose 50% of initial activity in the presence of 8 vol% butanol (Fig. S5†).^{10,11} Hence, with this new improved process stability and high activity (Table 1), PDC-Var. 2 rises as a better candidate than any PDCs reported to date and holds promise for the application of *in vitro* butanol production at its solubility limit.

In conclusion, we demonstrated that organic solvent-stable PDC variants could be found only by developing a screening platform focused on improved thermostability. In general, developing a screening platform for improved thermostability is easier than for organic solvent tolerance, as some organic solvents may possess incompatibility issues with enzymatic assays, and general handling is more difficult. We expect our new thermostable PDC variant (PDC-Var. 2) to enhance the feasibility of consolidated bioprocessing approach for ethanol production in cellulolytic thermophilic organisms.^{3,5} Improved butanol stability of PDC-Var. 2 should drive forward the already developed *in vitro* butanol production, either *via* CoA dependent or proline-facilitated aldehyde condensation pathways (Scheme 1).^{10,11} Additionally, improved thermostability and stability in the presence of butanol of PDC-Var. 2 will facilitate simpler product removal at high temperatures in cell-free butanol synthesis.

Conflicts of interest

There are no conflicts to declare.

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