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Cell-Free Metabolic Engineering: Production of Chemicals by Minimized Reaction Cascades

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The limited supply of fossil resources demands the development of renewable alternatives to petroleum-based products. Here, biobased higher alcohols such as isobutanol are versatile platform molecules for the synthesis of chemical commodities and fuels. Currently, their fermentation-based production is limited by the low tolerance of microbial production systems to the end products and also by the low substrate flux into cell metabolism. We developed an innovative cell-free approach, utilizing an artificial minimized glycolytic reaction cascade that only requires one single coenzyme. Using this toolbox the cell-free production of ethanol and isobutanol from glucose was achieved. We also confirmed that these streamlined cascades functioned under conditions at which microbial production would have ceased. Our system can be extended to an array of industrially-relevant molecules. Application of solvent-tolerant biocatalysts potentially allows for high product yields, which significantly simplifies downstream product recovery.

Introduction

The development of sustainable, biomass-based production strategies is influenced by factors such as availability of cheap, nonfood biomass, its efficient depolymerization into key intermediates (i.e., sugars), and flexible, efficient technologies to convert such intermediate streams into chemical products that are cost-competitive with petroleum equivalents. Alcohols, such as ethanol and isobutanol, are excellent molecular platforms for the sustainable production of chemical commodities and fuels. Presently, biotechnological approaches for the conversion of biomass to alcohols focus on well-established microbial fermentation processes.[1–5]

However, the conditions of fermentation processes remain restricted to the physiological limits of cellular production systems. Key barriers for the cost-effective implementation of fermentation processes include the low tolerance to temperature fluctuations, elevated temperatures in general, and diverse solvent conditions, which can result in low conversion efficiencies and yields. Additionally, the multitude of cellular metabolic pathways can often lead to the unintended use of non-productive reaction pathways. Despite advances in genetic engineering, streamlining these metabolic networks for optimal product formation at an organism level is prohibitively difficult and due to the high complexity continues to be rather unpredictable.

A prominent example is the production of isobutanol using recombinant fermentation in *Escherichia coli*. Concentrations of as low as 1–2% (*v*/*v*) isobutanol can already induce toxic effects in the microbial production host, reducing both growth rates and precursor synthesis and resulting in extremely low product yields.[1,4,5] Additionally, the cost-effective pretreatment of biomass usually produces toxic or nonfermentable components that limit microbial growth and product yields.[6] Therefore, cell-based production strategies for isobutanol and other industrial chemicals have difficulties to compete economically with petroleum-derived equivalents.

The solution for this problem might be surprisingly simple: leave out the cells and exclusively employ purified biocatalysts. Consequently, cell-associated process barriers such as substrate or product toxicity or the undesired, substrate-induced redirection into an alternative metabolism pathway can be eliminated.[7,8] Due to their reduced molecular complexity and rapid adaptability to harsh industrial reaction conditions, designed biocatalytic processes are superior to their cellular counterparts. Indeed, the concept of cell-free synthesis was already introduced more than 100 years ago by Buchner, who accomplished ethanol production with crude yeast cell extracts.[9] Since then, enzymatic reactions have been established as valuable tools for organic synthesis, whereby most reactions comprise only one or two enzyme-catalyzed steps. Multistep reactions resembling natural pathways (>4 enzymes) are currently either being used for analytical purposes (e.g., for the identification of bottlenecks in cellular dihydroxyacetonephosphate synthesis[10,11]) or for the production of structurally complex,

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valuable compounds (such as isotope-labeled nucleotides[12,13]), but the utilization of these processes has only recently been recognized as a promising technique for chemical synthesis of cheap and renewable base chemicals.[8]

Since many natural metabolic pathways have been shaped by evolution to regenerate cofactors such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NAD(P)H), one particular issue of cell-free systems is the need for a closed cofactor balance in absence of the cell-metabolism. A particularly noteworthy example is the reconstruction of yeast Embden–Meyerhof-pathway by Scopes and coworkers which required a total of 13 enzymes and NAD(H) as well as well-defined concentrations of adenosine driphosphate (ADP) and ATP.[14] ATP, which accumulates in the absence of a viable cellular metabolism, is required for the initiation of glycolysis. To manage balanced ATP cycling, the hydrolysis of excess ATP had to be adjusted very carefully or eliminated by using highly toxic arsenate.

Consequently, to yield stable and technically feasible cell-free processes, it is essential to minimize the number of enzymes and eliminate ATP-driven reactions. This goal can be achieved by designing artificial in vitro pathways. The design of these non-natural metabolic pathways in a cell-free environment is thereby only restricted by thermodynamic limitations and enzyme performance. One recent example for a successful engineering approach to a cell-free pathway is the conversion of different sugar derivatives to molecular hydrogen by Zhang et al., who designed a novel reaction cycle based on the pentose phosphate pathway and demonstrated that their system has the necessary adaptation capability to react to changes of substrate or product requirements.[15–17]

We have now designed a completely artificial glycolytic reaction cascade for the conversion of glucose to pyruvate that is comprised of only four enzyme-catalyzed reactions, thereby also eliminating any phosphorylation requirements. The artificial pathway is completely redox balanced, and it requires only a single molecular shuttle (NAD^+). Pyruvate is a central intermediate from which molecules like ethanol or isobutanol can be produced with few additional enzymatic steps. The novel cell-free engineering approach allowed production of ethanol and isobutanol under reaction conditions that are prohibitive to any cell-based microbial equivalents. As our reaction cascade is designed as a general process, other products can be envisioned as future targets.

Results and Discussion

General pathway design

The production of pyruvate from glucose was achieved by a modified non-phosphorylative Entner-Doudoroff-Pathway (np-ED) derived from hyperthermophilic archaea.[18] One mole of glucose was converted into two moles pyruvate, coupled with the reduction of two NAD^+ equivalents (Figure 1). To eliminate phosphorylation and dephosphorylation steps of the natural np-ED pathway and thus reduce the number of required enzymes, we exploited the substrate promiscuity of an archaeal dihydroxy acid dehydratase[19] (DHAD) which catalyzes both the transformation of glycerate to pyruvate as well as the conversion of gluconate to 2-keto-3-desoxygluconate. The molecular efficiency of DHAD (see Figure 1) allows for the consolidated conversion of glucose to pyruvate with just four enzymes: glucose dehydrogenase[20] (GDH), gluconate/glycerate/dihydroxyacid dehydratase,[19] 2-keto-3-desoxygluconate aldolase[21] (KDGA), and glyceraldehyde dehydrogenase[22,23] (AlDH). AlDH together with DHAD redirects glyceraldehyde produced through aldol cleavage towards pyruvate formation. Enzymes of the cell-free reaction cascade were chosen based on their stability and selectivity. In general thermostable enzymes from thermophiles are preferred, as they are prone to tolerate higher process temperatures and higher solvent concentrations.[24,25] Thus, enhanced thermostability allows for increased reaction rates, a higher rate of substrate diffusion, lower viscosities, better phase separation, and decreased bacterial contamination of the reaction medium. As demands for substrate selectivity vary at different reaction stages, enzyme fidelity has to be selected accordingly. The substrate tolerance of the *Sulfolobus solfataricus* DHAD was reported recently.[19] We found that in its recombinant form the enzyme has a specific activity of 0.66 $U\,mg^{-1}$ for gluconate and 0.011 $U\,mg^{-1}$ for glycerate respectively. In the conversion of glucose to the key intermediate pyruvate, DHAD allows for parallel conversion of gluconate and glycerate (Figure 1). In contrast to DHAD, an AlDH was chosen that is specific for glyceraldehyde and does not accept other aldehydes such as acetaldehyde[22] or isobutyraldehyde, which are downstream reaction intermediates. These prerequisites were met by a NADPH-dependent aldehyde dehydrogenase that was able to convert only D-glyceraldehyde to D-glycerate with excellent selectivity. In order to minimize reaction complexity, the designed pathway was further consolidated to use the coenzyme NADH as the only electron carrier. Consequently, a directed evolution approach was used to engineer an AlDH variant with a greater activity for NADH.[26] Provided that subsequent reactions maintain redox-neutrality, pyruvate can potentially be converted to an array of industrial platform chemicals without the continuous addition of any electron shuttle.

Ethanol synthesis

To demonstrate the feasibility of the cell-free synthesis toolbox, glucose was converted to pyruvate using the enzyme cascade described above. In a subsequent two-step reaction pyruvate was converted to acetaldehyde and then to ethanol by action of pyruvate decarboxylase[27] (PDC) and alcohol dehydrogenase[28,29] (ADH). As no variants of PDC with thermophilic origin have been reported so far, the equivalent enzyme from the mesophilic bacterium *Zymomonas mobilis* was selected due to its relatively high thermal tolerance and activity. Despite its mesophilic origin, *Z. mobilis* PDC is thermostable up to 50 °C (see Table 1) which is in accord with the temperature range of more thermostable enzymes from thermophiles. Consequently, experiments were carried out at 50 °C. The six required enzymes were recombinantly expressed in *E. coli* and subjected

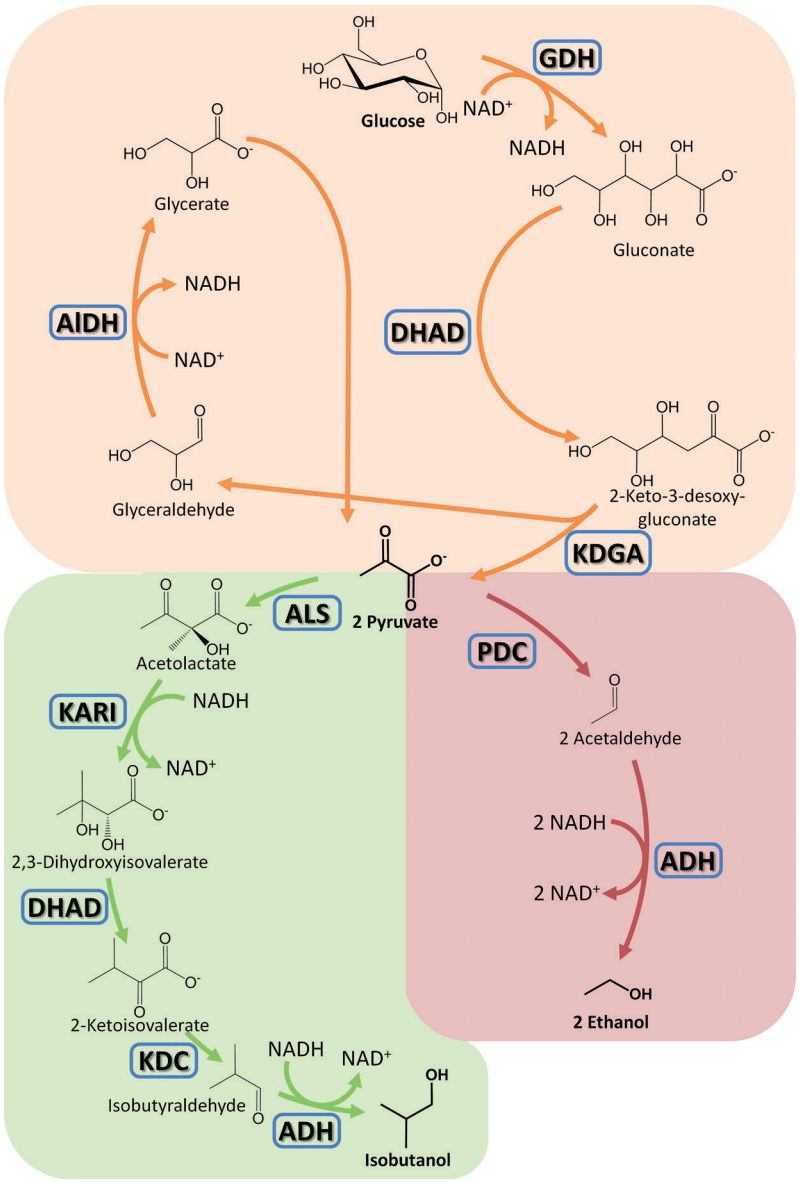

Figure 1. Schematic representation of cell-free reaction pathways to ethanol and isobutanol via minimized reaction cascades. In the first part of the reaction (top box) glucose is converted into two molecules of pyruvate. Depending on the desired final product and the enzymes applied, pyruvate can be either directed to ethanol (lower right box) or isobutanol synthesis (lower left box) in the second part of the reaction cascade. For clarity, protons and molecules of CO_2 and H_2O that are acquired or released in the reactions are not shown.

to different purification regimes. Using this set of enzymes, together with 5 mM NAD^+, we were able to convert 25 mM glucose to 28.7 mM ethanol (molar yield of 57.4%) in 19 h (Figure 2). Based on the initial substrate and coenzyme concentrations these results clearly demonstrate the successful recycling of NAD^+ and NADH. As the overall product yield exceeded 50%, it was also shown that the glyceraldehyde resulting from 2-keto-3-desoxygluconate cleavage was successfully redirected towards pyruvate. Next to ethanol and glucose, reaction intermediates such as gluconate, 2-keto-3-desoxygluconate, pyruvate, glycerate, and acetaldehyde were monitored during the course of the reaction. Especially for gluconate, the substrate of DHAD, a temporary accumulation of up to 8 mM was detected during the first 10 h of the reaction. In contrast, glycerate and acetaldehyde concentrations did not exceed 4 mM, while pyruvate was not detectable.

While residual intermediates generally accumulated at the end of the reaction cycle, the maximum gluconate concentration was measured between 8 and 10 h during the course of the reaction. Notably, undesired side-products such as lactate and acetate were not detected, indicating that the selected enzymes did provide the necessary substrate specificity. Although the enzyme-catalyzed reaction was not completed over the course of the experiment, the cumulative mass of all detectable intermediates and products gives a yield in excess of 80%.

Isobutanol synthesis

While specialized yeasts can tolerate ethanol at higher concentrations, longer-chain alcohols ($N \geq 4$) are incompatible with microbial physiology already at low concentrations.[32] Consequently, an alcohol as large as isobutanol, despite major cell-engineering efforts, has not been synthesized using microorganisms at a concentration higher than 2–2.5% *w/v*.[4]

While a non-natural isobutanol pathway has been described previously in the context of a cell-based system,[1] we have advanced the concept and converted pyruvate to isobutanol using only four additional enzymes (see Figure 1, Table 1) in a completely cell-free environment. Initially, two pyruvate molecules were joined by acetolactate synthase[30] (ALS) to yield acetolactate, which is further converted by ketolacid reductoisomerase[33] (KARI) resulting in the natural DHAD substrate dihydroxyisovalerate. DHAD then catalyzes the conversion of dihydroxyisovalerate into 2-ketoisovalerate.

The enzymes 2-ketoacid decarboxylase[27,31] (KDC) and an ADH[28,29] produced the final product, isobutanol, via isobutyraldehyde. Again the substrate tolerance of DHAD is exploited to minimize the total number of enzymes required.

Table 1. Enzymes used in the cell-free synthesis of ethanol and isobutanol[a].

Enzyme	EC[b]	Source organism	Activity[c] [U mg^{-1}]	Half-life [h]	*T*-Optimum [°C]	E_{50}[d] [% *v/v*]	I_{50}[e] [% *v/v*]
GDH	1.1.1.47	*S. solfataricus*	15	>24	70	30 (45 °C)	9 (45 °C)
DHAD	4.2.1.39	*S. solfataricus*	0.66 0.011 0.38	17	70	15 (50 °C)	4 (50 °C)
KDGA	4.2.1.14	*S. acidocaldarius*	4	>24	99[21]	15 (60 °C)	>12 (60 °C)[f]
AlDH	1.2.1.3	*Thermoplasma acidophilum*[g]	1	12	63[23]	13 (60 °C)	3 (50 °C)
PDC	4.1.1.1	*Z. mobilis*	64	22	50	20 (50 °C)	8 (45 °C)
ADH	1.1.1.1	*Geobacillus stearothermophilus*	210 83	>24	>60[28]	25 (50 °C)	5 (50 °C)
ALS	2.2.1.6	*Bacillus subtilis*	30	12	37[30]	n.d.	4 (50 °C)
KARI	1.1.1.86	*Meiothermus ruber*	0.7	34	55	n.d.	8 (40 °C)
KDC	4.1.1.72	*Lactococcus lactis*	150	>24	50[31]	n.d.	4 (45 °C)

[a] For details concerning cloning and expression see methods section. Activity and half-life measurements were taken at $T = 50$ °C. [b] Enzyme classification number. [c] Activity for natural substrates, DHAD for gluconate, glycerate and dihydroxyisovalerate, ADH for acetaldehyde and isobutyraldehyde (resp.) as substrates. [d] E_{50}: Ethanol concentration which causes loss of 50% activity. n.d.: not determined. [e] I_{50}: Isobutanol concentration which causes loss of 50% activity. [f] Above solubility. [g] Enzyme was engineered.

By analogy to ethanol production, the enzymes of the general pyruvate synthesis route differ from the following three biocatalysts with respect to thermal stability, solvent tolerance, and activity profiles (Table 1). To allow experimental comparison, the reaction conditions remained the same as described previously. Measurements indicated that 19.1 mM glucose was converted to 10.3 mM isobutanol within 23 h, which corresponds to a molar yield of 53% (Figure 3). During the first 10 h of the reaction, the product formation rate was 0.7 mM h^{-1}, which is similar to the ethanol formation rate of 2.2 mM h^{-1} (2 mol of ethanol instead of 1 mol of isobutanol is produced from 1 mol glucose). In contrast to the ethanol synthesis, only a minor accumulation of the DHAD substrates gluconate and glycerate was detected, resulting in a maximum concentration of 1.8 mM for

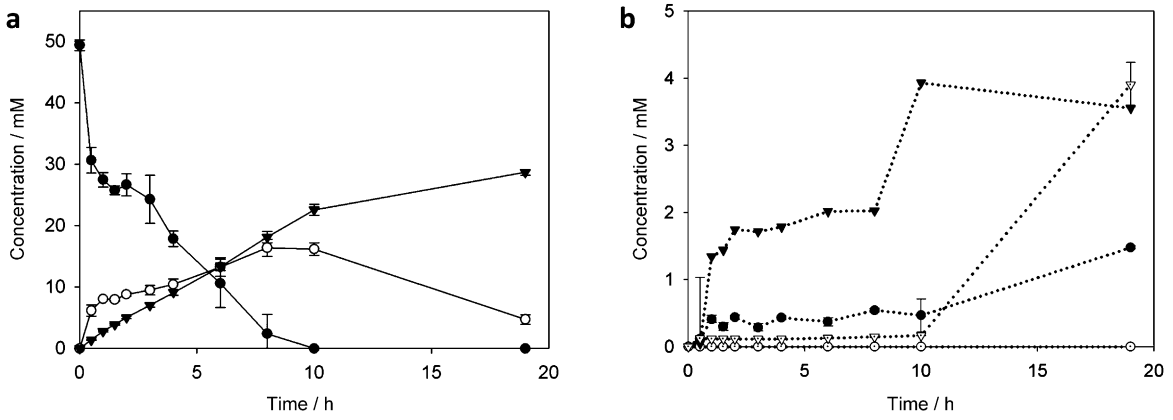

Figure 2. Cell-free synthesis of ethanol. a) Intermediates in concentrations >5 mM; ●: glucose, ○: gluconate, ▼: ethanol. b) Intermediates in concentrations <5 mM; ●: KDG, ○: pyruvate, ▼: glycerate, ▽: acetaldehyde. Note that the concentration of glucose, gluconate and KDG was duplicated to allow for a better comparison with the ethanol concentration (1 mol glucose is converted to 2 mol ethanol). All data points represent average values from three independent experiments.

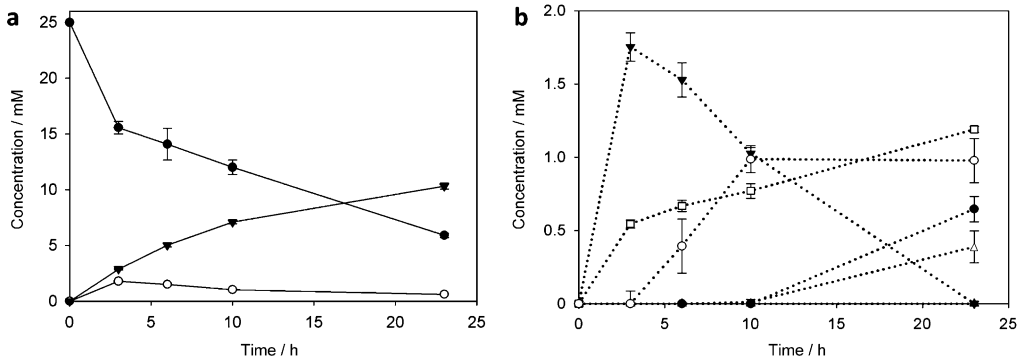

Figure 3. Cell-free synthesis of isobutanol. a) Intermediates in concentrations >2 mM; ●: glucose, ○: gluconate, ▼: isobutanol. b) Intermediates in concentrations <2 mM; ●: KDG, △: pyruvate, ▼: glycerate, □: isobutyraldehyde; ○: KIV. DHIV could not be detected at all. All data points represent average values from three independent experiments.

each of these intermediates. Additional reaction intermediates such as 2-keto-3-desoxygluconate, pyruvate, 2-ketoisovalerate, and isobutyraldehyde were measured at low concentrations (maximum 1.2 mM) that slowly increased towards the end of the measurement. Again substrate conversion was not completed within the monitored time. As with cell-free ethanol biosynthesis, quantification of all detectable intermediates gave a yield of 80%.

Solvent tolerance

A key characteristic of cell-free systems is their pronounced tolerance to the presence of higher alcohols. To evaluate solvent tolerance of our artificial enzyme cascade, glucose conversion to ethanol was conducted in the presence of increasing isobutanol concentrations (Figure 4). In contrast to microbial cells, where minor isobutanol concentrations (ca. 1% *v/v*) already result in a loss of productivity, presumably through the loss of membrane integrity,[5] cell-free ethanol productivity and reaction kinetics were not significantly affected by isobutanol concentrations up to 4% (*v/v*). Only in the presence of 6% (*v/v*) isobutanol, did the ethanol productivity rapidly decline (1.4 mM ethanol in 8 h). This demonstrates that cell-free processes have the potential to tolerate much higher solvent concentrations than equivalent whole-cell systems. Based on our current data AlDH has the lowest solvent tolerance, as 3% (*v/v*) isobutanol already induce adverse effects on activity. In contrast, KDGA remains completely active even in a two-phase isobutanol/water system, which forms spontaneously at product concentrations above 12% (*v/v*)[34] (see Table 1). As shown for an engineered transaminase, which remains active in a reaction medium containing 50% DMSO,[35] such shortcomings can be addressed by engineering of the respective protein. In comparison, there is neither a successful example nor a straightforward technology in place to engineer an entire cell for solvent tolerance. It is expected, that all enzymes utilized in our cell-free pathways can be engineered to be as solvent tolerant as KDGA or can be replaced by a stable naturally occurring equivalent, so that isobutanol production can be envisioned in a two-phase system. Product recovery by a simple phase separation would significantly simplify the downstream processing[36] and, while conceivable with a cell-free system, it is highly unlikely to be realized by microbial fermentation.

Conclusions

The stability and minimized complexity of our cell-free system eliminate the barriers of current cell-based production, which hamper the wider industrial exploitation of bio-based platform chemicals. Pyruvate is a central intermediate, which may serve as a starting point for cell-free biosynthesis of other commodity compounds. The enzymatic approach demonstrated here has been minimized in the number of required enzymes and coenzymes and therefore it has the potential to serve as a next generation bio-production system.

Substrate and product concentrations in the herein described experiments are relatively low. For allowing easy product separation, which is a prerequisite for an economically feasible process, the product concentration should be increased above the solubility limit, which for isobutanol is 1.28 M at 20 °C (ca. 95 g L^{-1}). Although the product solubility can be reduced by increasing the process temperature and adjusting the salt concentrations, an increase of substrate concentration (and thereby product concentration) is essential. As 1 mol glucose is converted to 1 mol isobutanol in our system, substrate concentrations have to be chosen at the desired end concentration (230 g L^{-1} glucose) or higher. Furthermore, a continuously running process using a constant substrate feed (glucose syrup) and product removal (organic phase) would be advantageous, given that the enzymes and cofactors could be retained, for example, by immobilization.

Ongoing molecular optimization of individual enzymes allows for iterative improvements and extension of the presented cell-free production systems with a particular focus on activity, thermal stability and solvent tolerance. In addition, the resistance to the inhibitors that are present when hydrolyzed lignocellulosic biomass is used as feedstock can be addressed by enzyme engineering, whereas these inhibitors can be detrimental to cell-based methods.

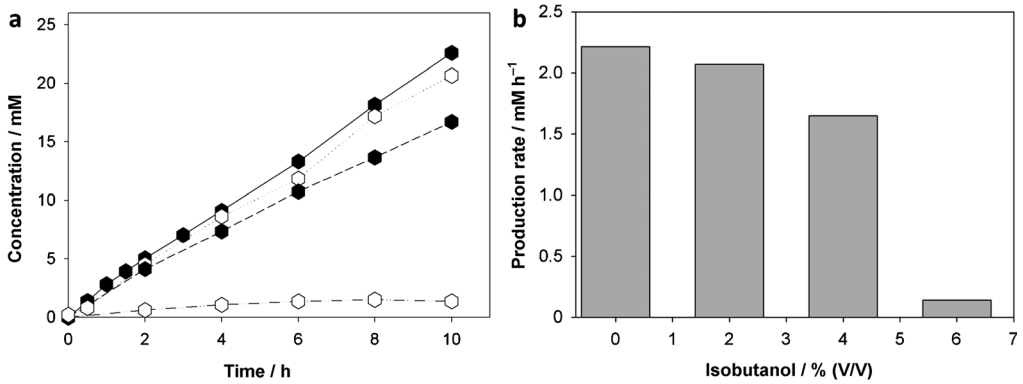

Figure 4. Ethanol production at different isobutanol concentrations. a) ⬢ ——: 0% isobutanol; ⬡, •••••: 2% isobutanol; ⬢, – – –: 4% isobutanol; ⬡, – – –: 6% isobutanol. b) ethanol production rate (mM h^{-1}) plotted against isobutanol concentration.

Experimental Section

Reagents

Restriction enzymes, Klenow fragment, T4 ligase and T4 kinase were purchased from New England Biolabs (Frankfurt, Germany). Phusion polymerase was from Finnzymes (Espoo, Finland), desoxynucleotides from Rapidozym (Berlin, Germany). All enzymes were used according to the manufacturers' recommendations, applying the provided buffer solutions. Oligonucleotides were ordered from Thermo Scientific (Ulm, Germany). Full-length genes were synthesized by Geneart (Regensburg, Germany), with optimized *E. coli* codon usage, and delivered in the company's standard plasmids. Porcine heart lactate dehydrogenase (LDH) was bought from Serva (Heidelberg, Germany), *Aspergillus niger* glucose oxidase and horseradish peroxidase from Sigma–Aldrich (Munich, Germany). All chemicals were, unless otherwise stated, purchased in analytical grade from Sigma–Aldrich, Carl Roth (Karlsruhe, Germany), Serva Electrophoresis and Merck (Darmstadt, Germany).

Strains and Plasmids

E. coli BL21(DE3) (F^- *ompT hsdSB* (rB^- mB^-) *gal dcm* (DE3)) was purchased from Novagen (Nottingham, UK), *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacIqZΔM15* Tn10 (Tetr)]) from Stratagene (Waldbronn, Germany). pET28a-DNA was provided by Novagen.

Vector construction

Plasmids pCBR, pCBRHisN and pCBRHisC were constructed on the basis of pET28a (Novagen). DNA-sequences (see Table 2) for the corresponding new multiple cloning sites were synthesized (Geneart, Regensburg, Germany) and cloned into pET28a via *Xba*I/*Bam*HI (pCBR), *Nde*I/*Eco*RI (pCBRHisN) or *Xba*I/*Bpu*1102I (pCBRHisC), thereby replacing the existing multiple cloning site with a new restriction site containing a *Bfu*AI- and a *Bsa*I-sequence and, in case of pCBR and pCBRHisN, a stop codon. The three new vectors allow the simultaneous cloning of any gene using the same restriction sites, enabling the user to express the respective gene without or with an N- or C-terminal His-tag, whereby a stop codon must not be attached at the 3′-end of the gene. Vector-DNA was first restricted with *Bsa*I, followed by blunt end generation with Klenow fragment. Afterwards, the linearized plasmids were digested with *Bfu*AI, generating a 5′-overhang. Genes were amplified using the Geneart vectors as templates and the corresponding oligonucleotides (Table 3). After PCR, DNA fragments were digested with *Bsa*I, 3′-phosphorylated (T4 kinase) and subsequently ligated into the appropriate vectors. In some cases, phosphorylation could be replaced by digestion using *Psi*I. Plasmids were transformed into *E. coli* as described elsewhere.[37] Sequence analysis was performed by GATC Biotech (Konstanz, Germany). pET28a-HisN-LlKdcA was cloned according to Gocke et al.[31]

Table 2. Vector multiple cloning sites.

Name	DNA-Sequence (5′→3′)
pCBR	ATATATATATTCTAGAAATAATTTTGTTTAACTTTAAGAA GGAGATATACATATGATGCAGGTATATATATATTAATAG AGACCTCCTCGGATCCATATATATAT
pCBRHisN	ATATATATATCATATGATGCAGGTATATATATATTAATAG AGACCTCCTCGAATTCATATATATAT
pCBRHisC	ATATATATATTCTAGAAATAATTTTGTTTAACTTTAAGAA GGAGATATACATATGATGCAGGTATATATATATAGCGG GAGACCTGTGCTGGGCAGCAGCCACCACCACCACCACC ACTAATGAGATCCGGCTGCTAACAAAGCCCGAAAGGAA GCTGAGTTGGCTGCTGCCACCGCTGAGCATATATATAT

Table 3. Oligonucleotides.

Oligonucleotide	Gene amplified	Oligonucleotide sequence (5′→3′)
SsGDH_for	*S. solfataricus* glucose dehydrogenase	CAGCAAGGTCTCACATAT GAAAGCCATTATTGTGAA ACCTCCG
SsGDH_rev	*S. solfataricus* glucose dehydrogenase	TTCCCACAGAATACGAAT TTTGATTTCGC
SsDHAD_for	*S. solfataricus* dihydroxyacid dehydratase	CAGCAAGGTCTCACATAT GCCTGCAAAACTGAATAG CCC
SsDHAD_rev	*S. solfataricus* dihydroxyacid dehydratase	TGCCGGACGGGTAACT GC
SaKDGA_for	*S. acidocaldarius* KDG aldolase	CAGCAAGGTCTCACATAT GGAAATTATTAGCCCGAT TATTACCC
SaKDGA_rev	*S. acidocaldarius* KDG aldolase	ATGAACCAGTTCCTGAAT TTTGCG
TaAlDH_for	*T. acidophilum* glyceraldehyde dehydrogenase	CAGCAAGGTCTCACATAT GGATACCAAACTGTATAT TGATGGC
TaAlDH_rev	*T. acidophilum* glyceraldehyde dehydrogenase	CTGAAACAGGTCATCACG AACG
MrKARI_for	*M. ruber* ketolacid eductoisomerase	CAGCAACGTCTCGCATAT GAAGATTTACTACGACCA GGACGCAG
MrKARI_rev	*M. ruber* ketolacid reductoisomerase	GCTACCGACCTCTTCCTT CGTGAAC

Enzyme expression

Enzyme expression was performed using *E. coli* BL21(DE3) or BL21 Rosetta(DE3)-pLysS as host strains, either in shaking flask cultures or in a 10 L Biostat Cplus bioreactor (Sartorius Stedim, Göttingen, Germany). All media were supplemented with 30–50 $\mu g\,mL^{-1}$ kanamycin. GDH and DHAD were expressed in LB medium, acetolactate synthase in TB medium. After inoculation cells were grown at 37 °C to an optical density at 600 nm of 0.6, induced with 1 mM IPTG and the temperature lowered to 16–20 °C for 16–24 h expression. KDGA and AlDH were expressed according to the fed-batch cultivation method of Neubauer et al.[38] at 37 °C. After inoculation cells were grown for 24 h and induced with 1 mM IPTG. Enzyme expression was performed for 24 or 30 h, respectively. KDC expression was performed for 22 h at 30 °C in batch mode using Zyp-5052[39] as a medium. KARI was expressed in a batch fermentation using TB medium. Cells were grown at 37 °C to an optical density of 5.2 and induced by the addition of 0.5 mM IPTG. Afterwards, expression was performed for 24 h at 20 °C.

Enzyme purification

All protein purification steps were performed using an ÄKTA UPC-900 FPLC-system (GE Healthcare, Freiburg, Germany), equipped

with HiTrap FF-, H*i*Prep 26/10 Desalting- and HiTrap Q-Sepharose FF-columns (GE Healthcare). Cell lysates were prepared with a Basic-Z Cell Disruptor (Constant Systems, Northants, UK), cell debris was removed by centrifugation at 35,000×g and 4 °C for 30 min (Sorvall RC6+, SS-34 rotor, Thermo Scientific). For lyophilization an Alpha 2–4 LD Plus freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany) was used. GDH and DHAD were purified by heat denaturation (30 min at 70 °C, respectively). GDH was subsequently freeze-dried (SpeedVac Plus, Thermo Scientific), DHAD concentrated using a stirred Amicon cell (Milipore, Darmstadt, Germany) and either stored at −80 °C or directly applied to experiments. KDGA, AlDH and KDC were purified as previously described[21,23,31] and stored as lyophilisates. ALS and KARI were purified via IMAC using 25 or 50 mM HEPES, pH 7. Elution was achieved with 500 mM imidazol. Enzymes were desalted and stored as a liquid stock (ALS) or lyophilisate (KARI).

Protein determination

Protein concentration was determined with the Roti-Nanoquant reagent (Carl Roth) according to the manufacturer's recommendations using bovine serum albumin as a standard.

SDS-PAGE

Protein samples were analyzed as described by Laemmli[40] using a Mini-PROTEAN system from Bio-Rad (Munich, Germany).

Enzyme assays

All photometrical enzyme assays were performed in microtiter plate format using a Thermo Scientific Multiskan or Varioskan photometer. When necessary, reaction mixtures were incubated in a waterbath (Julabo, Seelbach, Germany) for accurate temperature control. Buffers were prepared according to Stoll,[41] adjusting the pH to the corresponding temperature. Reactions using NAD^+ or NADH as coenzymes were followed at 340 nm (molar extinction coefficient NADH = 6.22 L mmol^{-1} cm^{-1}) and the glucose concentrations were measured at 418 nm and 480 nm as indicated.[27] One unit of enzyme activity is defined as the amount of enzyme necessary to convert 1 μmol substrate per minute. In addition to the standard reaction conditions described below, enzyme activity was tested under reaction conditions (100 mM HEPES, pH 7, 2.5 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate) prior to alcohol synthesis experiments.

GDH activity: GDH activity was assayed at 50 °C by oxidizing D-glucose to gluconate, whereby the coenzyme NAD^+ is reduced to NADH. Assay mixture contained 50 mM HEPES (pH 7), 2 mM NAD^+ and 50 mM D-glucose.[20]

DHAD activity: DHAD activity was measured by an indirect assay. The assay mixture containing DHAD, 20 mM substrate and 100 mM HEPES (pH 7) was incubated at 50 °C. Afterwards the conversion of glycerate to pyruvate, gluconate to 2-keto-3-desoxygluconate or 2,3-dihydroxyisovalerate to 2-ketoisovalerate, respectively, was determined via HPLC as described below.

KDGA activity: KDGA activity was followed in cleavage direction at 50 °C. Reaction mixture contained 50 mM HEPES (pH 7), 0.1 mM thiamine pyrophosphate, 2.5 mM $MgCl_2$, 20 U PDC and 10 mM KDG. KDG cleavage was followed by HPLC as described below.

AlDH activity: AlDH activity was assayed at 50 °C by oxidizing D-glyceraldeyde to glycerate, whereby the coenzyme NAD^+ is reduced to NADH. Assay mixture contained 50 mM HEPES (pH 7), 2.5 mM $MgCl_2$, 4 mM NAD^+ and 5 mM D,L-glyceraldehyde.[23]

ALS activity: ALS activity was determined by following pyruvate consumption at 50 °C. Reaction mixtures contained 25 mM HEPES (pH 7), 0.1 mM thiamine pyrophosphate, 2.5 mM $MgCl_2$, 15 mM sodium pyruvate. Pyruvate concentration in the samples was determined via lactate dehydrogenase as described elsewhere.[21]

KARI activity: KARI activity was assayed by following the NADH consumption connected to the conversion of acetolactate to 2,3-dihydroxyisovalerate at 50 °C. The assay mixture contained 5 mM acetolactate, 0.3 mM NADH, 10 mM $MgCl_2$ and 50 mM HEPES, pH 7.

KDC activity: KDC activity was assayed by following the decarboxylation of 2-ketoisovalerate to isobutyraldehyde at 50 °C and 340 nm. Assay mixture contained 50 mM HEPES (pH 7), 0.1 mM thiamine pyrophosphate, 2.5 mM $MgCl_2$ and 60 mM 2-ketoisovalerate. Decarboxylation rate was calculated using the molar extinction coefficient of 2-ketoisovalerate ($\varepsilon = 0.017$ L mmol^{-1} cm^{-1}).[27]

ADH activity: ADH activity was determined by following the NADH-dependent reduction of isobutyraldehyde to isobutanol at 50 °C. Assay mixture contained 10 mM HEPES (pH 7.2), 5 mM isobutyraldehyde and 0.3 mM NADH.

Glucose analysis: Glucose oxidase was used for the quantification of glucose. Assay mixture contained 20 mM potassium phosphate (pH 6), 0.75 mM 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), 2 U glucose oxidase and 0.1 U peroxidase. After the addition of samples the reaction mixture was incubated for 30 min at 30 °C and the extinction at 418 and 480 nm measured. Assay calibration was performed using defined glucose standard solutions.[42]

GC–FID analysis

Isobutyraldehyde and isobutanol or acetaldehyde and ethanol were quantified by GC–FID using a Thermo Scientific Trace GC Ultra, equipped with a flame ionization detector (FID) and a Headspace Tri Plus autosampler. Alcohol and aldehyde compounds were separated by a StabilWax column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness; Restek, Bellefonte, USA), whereby helium (0.8 or 1.2 mL min^{-1}) was used as the carrier gas. The oven temperature was programmed to be held at 50 °C for 2 min, raised with a gradient 10 °C min^{-1} to 150 °C and held for 1 min. Injector and detector were kept at 200 °C. Samples were incubated prior to injection at 40 °C for 15 min. Injection was done in the split mode with a flow of 10 mL min^{-1}, injecting 700 μL using headspace mode.

HPLC analysis

Gluconate, 2-keto-3-desoxygluconate, pyruvate, glycerate, 2,3-dihydroxyisovalerate and 2-ketoisovalerate were separated and quantified by HPLC, using an Ultimate-3000 HPLC system (Dionex, Idstein, Germany), equipped with autosampler and a diode-array detector. Chromatographic separation of gluconate, 2-keto-3-desoxygluconate, pyruvate and glycerate was achieved on a Metrosep A Supp10–250/40 column (250 mm, particle size 4.6 μm; Metrohm, Filderstadt, Germany) at 65 °C by isocratic elution with 12 mM ammonium bicarbonate (pH 10), followed by a washing step with 30 mM sodium carbonate (pH 10.4). Mobile phase flow was adjusted to 0.2 mL min^{-1}. 2,3-dihydroxyisovalerate and 2-ketoisovalerate were separated using a Nucleogel Sugar 810H column (300 mm, 7.8 mm internal diameter; Macherey–Nagel, Düren, Germany) at 60 °C by isocratic elution with 3 mM H_2SO_4 (pH 2.2). Mobile phase flow was adjusted to 0.6 mL min^{-1}. Sample volume was 10 μL in

each case. System calibration was performed using external standards of each of the abovementioned intermediates. Samples were prepared by filtration (10 kDa MWCO, modified PES; VWR, Darmstadt, Germany) and diluted.

Alcohol biosynthesis

All reactions were set up in 20 mL GC vials. Reaction mixtures contained 100 mM HEPES (pH 7 at 50 °C), 0.1 mM thiamine pyrophosphate, 2.5 mM $MgCl_2$, 25 mM D-glucose and 5 mM NAD^+. Enzymes were added as follows: GDH: 6 U, DHAD: 20 U for ethanol synthesis and 30 U for isobutanol synthesis, all other enzymes: 10 U. Control reactions were performed either without enzymes or without D-glucose. Reaction mixtures were placed in a water bath at 50 °C and gently stirred at 100 rpm.

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Keywords: alcohols · biocatalysis · biosynthesis · biotechnology · enzymes

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