Towards Cell-Free Isobutanol Production: Development of a Novel Immobilized Enzyme System

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Producing fuels and chemical intermediates with cell cultures is severely limited by low product concentrations (≤0.2%(v/v)) due to feedback inhibition, cell instability, and lack of economical product recovery processes. We have developed an alternate simplified production scheme based on a cell-free immobilized enzyme system. Two immobilized enzymes (keto-acid decarboxylase (KdcA) and alcohol dehydrogenase (ADH)) and one enzyme in solution (formate dehydrogenase (FDH) for NADH recycle) produced isobutanol titers 8 to 20 times higher than the highest reported titers with S. cerevisiae *on a mol/mol basis. These high conversion rates and low protein leaching were achieved by covalent immobilization of enzymes (ADH) and enzyme fusions (fKdcA) on methacrylate resin. The new enzyme system without* in situ *removal of isobutanol achieved a 55% conversion of ketoisovaleric acid to isobutanol at a concentration of 0.135 (mole isobutanol produced for each mole ketoisovaleric acid consumed). Further increasing titer will require continuous removal of the isobutanol using an in situ recovery system.* © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 32:66–73, 2016
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Introduction

The toxic effects of higher order alcohols on the cell (*Escherichia coli*, *Clostridium*, *Saccharomyces cerevisiae*, brown microalgae, blue-green algae, and others) has been observed at concentrations as low as 2% (v/v) for ethanol[1] and 0.2% (v/v) for isobutanol.[2] Isobutanol can impact many of the interconnected metabolic pathways within the cell.[3] To overcome feedback inhibition, toxicity, low titers, and other problems associated with in vivo synthesis of biofuels, researchers have investigated cell-free in vitro systems. Zhang et al. proposed in vitro systems for producing hydrogen, butanols, and other biofuels.[4–6] Isolating the enzymes directly responsible for the production of a bioderived fuel allows one to focus on optimizing the reaction without the need to optimize a complex full live cell system. Guterl et al. developed a reaction cascade to produce isobutanol using nine purified enzymes.[7] These systems demonstrate the potential of in vitro synthesis of biofuels. Such in vitro cascades with enzymes free in solution, however, have limitations: recycling a cofactor,[8] enzyme stability[5] and recovery of the purified desired product[9] are all challenging. The immobilized system presented here focuses on addressing the problem of enzyme stability and removal of the desired product. An immobilized reaction system allows for the liquid reaction mixture to be easily removed from the covalently immobilized enzymes. This system renders alcohol/water separation easier because the solution is protein-free.

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Enzymes that are not able to retain activity when immobilized require molecular engineering to improve their activity and stability. Specifically, when keto-acid decarboxylase (KdcA) [EC number 4.1.1.72] is overexpressed and purified, it self-aggregates; this results in a loss of activity. One strategy for engineering an enzyme is to create specific modifications, or site specific mutagenesis through rational or semi-rational design.[10] This method, however, requires a detailed understanding of the link between the protein sequence and stability and activity. Also, a detailed understanding of the relationship between protein structure and function, or kinetic activity, is often unavailable. Highly stable, soluble proteins like super folded green florescent protein (GFP)[11] and stabilized maltose binding domain (MBD)[12] have been fused to proteins to increase their solubility. By increasing the solubility of the desired proteins, their expression was increased.[11,12] Thus, GFP or MBD could be coupled to an unstable protein to increase solubility and possibly lessen protein-protein self-interactions.[11,12] Therefore, we chose this approach to stabilize immobilized KdcA and retain its activity. Previous work has shown that alcohol dehydrogenase (ADH) [EC number 1.1.1.1] can be immobilized onto different surfaces and retain its enzymatic activity.[9] Additionally, directed evolution can be employed to increase the activity of the enzymes. For example, the Arnold lab engineered ADH to have a 40-fold increase in its enzymatic activity versus wild type.[13] These active mutated enzymes have the potential to greatly increase the production of an immobilized enzyme system; however, here, we have used commercially available wild-type ADH from *Saccharomyces cerevisiae*.

In this work, we focused on an immobilized enzyme system and characterized and optimized the activity of two enzymes

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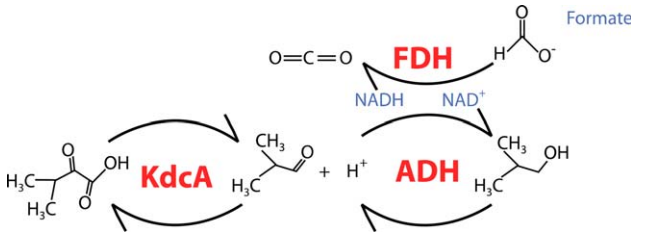

Figure 1. Two-step enzymatic production of isobutanol with cofactor recycling.

(KdcA, ADH) (Figure 1) involved in the conversion of ketoisovaleric acid, a nontoxic cellular intermediate, via isobutyraldehyde to isobutanol, a valuable product that is toxic to cells. Additionally, we explored how isobutanol concentration and temperature affected the in vitro kinetic activity of the enzymes. These two factors can have dramatic effects on enzyme stability, reaction reversibility, and enzyme activity. Hence we assume that these in vitro stability experiments will act as a surrogate for immobilized stability. Fusion proteins, developed to assess stability of KdcA, were overexpressed and purified. They exhibited higher conversion kinetics and increased stability when immobilized compared with the mutants developed after one round of screening. Finally by stabilizing KdcA, a fully immobilized two-step production scheme to convert ketoisovaleric acid to isobutanol was demonstrated.

Methods

Materials

All materials and reagents were used as received. All buffers were at physiological pH and room temperature unless specified otherwise. Alcohol dehydrogenase from *Saccharomyces cerevisiae* [A3263] (ADH, 146.8 kDa, pI 5.4), β-nicotinamide adenine dinucleotide (NADH), formate dehydrogenase from *Candida boidinii,* (FDH), ketoisovaleric acid, formate, isobutyraldehyde, thiamine pyrophosphate (ThDP), and buffer salts were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI). Methacrylate resin (Relizyme HFA403/S) [100–300 micron] was purchased from Itochu Chemicals America (White Plains, NY). Gas chromatography column: 25346 SUPELCO SPB®-5 Capillary GC Column L × I.D. 15 m × 0.53 mm, df 5.00 μm was purchased from SUPELCO (Milwaukee, WI).

Fusion protein development

A vector with the genes for super folded GFP and stabilized MBD was obtained from Dr. Marlene Belfort's research group, University at Albany, State University of New York, Albany, NY. Primers (Table 1) were created to amplify the GFP, MBD, and KdcA genes. The initial gene fragments were PCR amplified from their respective vectors. The full fusion protein gene was then PCR amplified using the 22 base pair overlapping sequence designed in the primers for the fused protein (GFP or MBD) gene and the KdcA gene. The full genes were digested with *XhoI* and *NdeI* and ligated with digested pet17b vector using *T4* DNA ligase. The ligation mixtures were transformed into chemically competent Top10 or BL21(DE3) cells. The genes were fully sequenced to verify the presence of the KdcA gene.

96-Well plate KdcA fusion protein activity assay

The reaction mixture contained: 125 μL buffer A (50 mM potassium phosphate buffer, pH 6.8, 2.5 mM MgSO4, 0.1 mM ThDP), and 25 μL 1 mg KdcA or fusion protein/mL buffer A. The assay was started by addition of 50 μL ketoisovaleric acid 120 mM in buffer A (final concentration 30 mM) to each well. Kinetic activity was followed (37°C) by observing the consumption of ketoisovaleric acid, as demonstrated by the decrease in absorbance at 340 nm over time.

Fusion protein analysis

Each fusion protein was expressed in 100 mL of AI media.[14] His-GFP-KdcA (HGK) and MBD-KdcA-His (MKH) were purified using Ni-NTA; MBD-KdcA (MK) was purified using immobilized amylose chromatography. Kinetic constants were determined by assaying the activity of the fusion proteins with a range of ketoisovaleric acid concentration (0–250 mM). The stability of the enzymes was tested using the procedure above. The HGK fusion protein was assayed at three protein concentrations (5, 2, and 1 mg/mL) and two replicates each ($n = 6$). The MK fusion protein was assayed at two protein concentrations (2 and 1 mg/mL) and two replicates ($n = 4$). The MKH fusion protein was assayed at two protein concentrations (2 and 1 mg/mL) and two replicates ($n = 4$). The wild-type KdcA was assayed at one protein concentration (1 mg/mL) and two replicates ($n = 2$).

Temperature stability of KdcA

Solutions (2 mL) 100 μg/mL KdcA (overexpressed and purified) were heated to various temperatures listed below

Table 1. Fusion Protein Primers*

Primer	Nucleotide Sequence	Melting Temp °C†	% GC
His_GFP_Fusion_5′	AGC TGA CAT ATG GGC TCC TCT CAT CAT CAT CAC CAC CAC TCA TCA GGC AGC AAA GGA GAA GAA CTT TTC ACT G	70	40
GFP_KdcA_Fusion_3′	[CCC GCG CCC TGG AAA TAC AGG T]TC TCG TTG TTG TTA TTG TTA TTG TTG TTG TTC GAG CTC GAA TTT TTG TAG AGC TCA TCC ATG CCA TG	70	46
KdcA_Fusion_5′	[ACC TGT ATT TCC AGG GCG CGG G]TT ACA CCG TTG GCG AC	70	67
KdcA_GFP_Fusion_3′	AGC TGA GGA TCC TCA TTT ATT CTG TTC GGC AAA C	72	33
His_MBD_Fusion_5′	AGC TGA CAT ATG GGC TCC TCT CAT CAT CAT CAC CAC CAC TCA TCA GGC AAA ATC GAA GAA GGT AAA CTG GTA ATC	72	33
MBD_KdcA_Fusion_3′	[CCC GCG CCC TGG AAA TAC AGG T]TC TCG TTG TTG TTA TTG TTA TTG TTG TTG TTG	72	29
NdeI_His_5′	AGC TGA CAT ATG GGC TCC TCT CAT C	74	52

*Overlap (22 bp, melt 72°C, 64% GC) [sequence inside square brackets].
†Temperature calculated based on GC content.

for 10 min in glass vials using a heating block (Isotemp 125D, Fisher Scientific, Pittsburgh, PA). The vials were allowed to cool to room temperature in ambient conditions. Temperatures for KdcA: room temperature (22°C), 35°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C. Once cooled, 200 μL aliquots of the enzyme solutions were each pipetted into eight wells of a 96-well plates and assayed. Fifty microliters of ketoisovaleric acid 120 mM in buffer A (50 mM potassium phosphate buffer, pH 6.8, 2.5 mM MgSO4, 0.1 mM ThDP) (final concentration 30 mM) was added to each well to start the reaction. KdcA converts ketoisovaleric acid (ABS 340 nm) to isobutyraldehyde. Kinetic activity was tracked by observing the decrease in absorbance over time.

Alcohol tolerance of KdcA

0, 0.1, 0.6, 1, 2, 6, 10, 16, and 20 μL isobutanol (0%, 0.1%, 0.3%, 0.5%, 1%, 3%, 5%, 8%, and 10% isobutanol); 90, 89.9, 89.4, 89, 88, 84, 80, 74, or 70 μL buffer A, respectively; and 60 μL 1 mg KdcA/mL buffer A (final concentration 300 μg/mL) were added to wells of the 96-well plate. 50 μL ketoisovaleric acid 120 mM in buffer A (final concentration 30 mM) was added to each well to start the reaction. KdcA converted ketoisovaleric acid to isobutyraldehyde; ketoisovaleric acid absorbs light at 340 nm and isobutyraldehyde does not. Kinetic activity was tracked by observing the decrease in absorbance over time.

One-step methacrylate resin immobilization

Amino-epoxy methacrylate resin (50 mg) was rehydrated on an end-over-end mixer with di-H_2O for 1 h, and then centrifuged and resuspended in 1 mL (3 mg of HGK, MK, or MKH/mL buffer A) protein solution for 12 h. Resin particles were centrifuged and the supernatant assayed at 280 nm to determine protein concentration. Amino-epoxy methacrylate resin was washed with fresh buffer, and then assayed according to the 96-well plate assay conditions described below. Kinetics of immobilized enzyme was compared with that for equivalent enzyme free in solution.

96-Well plate KdcA fusion protein activity assay

The reaction mixture contains: 125 μL buffer A (50 mM potassium phosphate buffer, pH 6.8, 2.5 mM MgSO4, 0.1 mM ThDP), and 25 μL 25 mg particles/mL buffer A. The assay was started by addition of 50 μL ketoisovaleric acid 120 mM in buffer A (final concentration 30 mM) to each well. Kinetic activity was followed (at 37°C) by observing the consumption of ketoisovaleric acid, as demonstrated by the decrease in absorbance at 340 nm over time.

Immobilization of KdcA and ADH for full immobilized reaction scheme

Amino-epoxy methacrylate resin (50 mg) (Resindion, Roma, Italia) was rehydrated on an end-over-end mixer with Di-H_2O for 1 h, and then centrifuged and resuspended in 1 mL (5 mg ADH/mL buffer A, 2, 1, or 0.5 mg KdcA/mL buffer A w/glycerol, or KdcA fusion (MKH)) protein solution for 12 h. Resin particles were centrifuged and the supernatant assayed at 280 nm to determine protein concentration. Amino-epoxy methacrylate resin was washed with fresh buffer, and then assayed according to the 96-well plate assay conditions described above. Conversion kinetics of the immobilized enzyme was compared with that of the equivalent enzyme free in solution.

ADH/amino-epoxy methacrylate resin and MKH/amino-epoxy methacrylate resin assay with cofactor recycling

Low concentration system mimicking our previous test conditions (above): 100 mg of amino-epoxy methacrylate resin with immobilized ADH and 100 mg of amino-epoxy methacrylate resin with immobilized MKH were resuspended in 1 mL of stock reaction mixture (64 μmol ketoisovaleric acid, 250 μg FDH, 1.2 μmol NADH in buffer A, and either 70 μmol of formate (with formate case) or equivalent volume of water (without formate case) was added to the reaction. Full reaction volume: 1 mL.

High concentration system developed to facilitate in situ removal of isobutanol: 100 mg of amino-epoxy methacrylate resin with immobilized ADH and 100 mg of amino-epoxy methacrylate resin with immobilized MKH were resuspended in 5 mL of stock reaction mixture (320 μmol ketoisovaleric acid, 250 μg FDH, 6 μmol NADH in buffer A, and 1,500 μmol of formate). Full reaction volume: 5 mL.

The mixtures were then placed in 1.5 mL Eppendorf tubes in an end-over-end mixer (Mini-Tube Rotator, Fisher Scientific, Pittsburg, PA) for 24 h. The contents of the reaction mixture before and after incubation with the resin were determined by gas chromatography (GC) analysis.

Results and Discussion

KdcA fusion protein development in solution

Super-folded GFP or previously stabilized MBD was genetically fused to wild-type KdcA, according to the procedure outlined above. GFP and MBD were selected because they have previously been shown to increase the solubility of proteins.[12,15] The enzyme is fused to the stabilizing proteins by a short flexible peptide linker with no protease sites that is coded at the genetic level and is present in the full fusion after it is expressed. In order to determine the impact of the fused proteins on enzyme kinetics and ascertain which fusion protein exhibited the greatest improvement in activity and stability over wild-type, enzymatic activity was tracked by observing the consumption of ketoisovaleric acid over time. Since the reaction is a one-to-one reaction with no significant side product, substrate consumption is inversely correlated with product formation. This relationship was independently confirmed using gas chromatography (GC) analysis of the product; the total amount of ketoisovaleric acid consumed was equal to the amount of product formed within the detection limit and error of the GC (0.1 mM and ± 0.5 mM, respectively). From these measurements, and the supporting GC data, we obtained how the total substrate consumption per time depends on substrate concentration (Figure 2).

From the kinetic activity data, K_M and k_{cat} were calculated (Table 2). K_M is the Michaelis constant and is defined as the concentration of the substrate at which half the maximum velocity of the rate is obtained. The enzymatic conversion or turnover frequency, k_{cat}, is defined as the max velocity of the reaction divided by the enzyme concentration. The maltose binding protein-keto-acid decarboxylase (MK) fusion showed the best substrate consumption over time; this

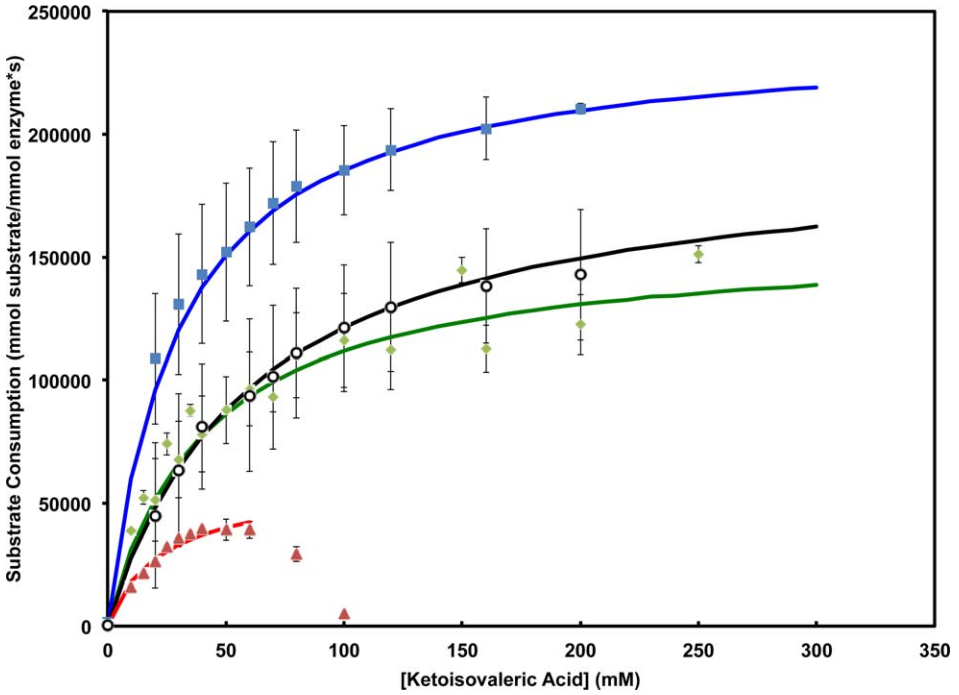

Figure 2. Ketoisovaleric acid specific activity for different fusion proteins as a function of substrate concentration.

Wild-type KdcA (▲) (n = 2), HGK(◆) (n = 6), MKH (○) (n = 4), and MK (■) (n = 2) substrate consumption. Fit lines are calculated using non-linear regression modeling of the Michaelis–Menten equation.

Table 2. Michaelis-Menten Kinetic Constants

	Wild-type	HGK	MK	MKH
K_M (mM)	22 ± 8	41 ± 10	30 ± 11	60 ± 30
k_{cat} (1/s) ($\times 10^4$)	5.8 ± 0.02	16 ± 2.5	24 ± 1.3	20 ± 0.3

enzyme construct had the highest enzymatic activity ($k_{cat} = 24 \pm 1.3 \times 10^4$ s^{-1}) (Table 2). The his-tagged fusion of keto-acid decarboxylase and green fluorescent protein (HGK) and maltose binding protein-keto-acid decarboxylase-his-tag fusion (MKH) had similar enzymatic activity ($k_{cat} = 16 \pm 2.5 \times 10^4$ s^{-1} and $20 \pm 0.3 \times 10^4$ s^{-1}, respectively). Wild-type KdcA had the lowest activity ($k_{cat} = 5.8 \pm 0.02 \times 10^4$ s^{-1}) and also exhibited substrate inhibition due to inactivation of the enzyme by the substrate (Figure 2). We found that the K_M increased from 22 mM to between 30 and 60 mM for the fusion proteins. The change in K_M is not statistically different. This small change is optimal; it means that the substrate does not need to be at a higher concentration to reach the maximum rate of conversion. Additionally, we report that the turnover frequency increased 2.5- to 4-fold over that for the wild-type.

By coupling a stable protein like GFP or MBD to KdcA, we were able to decrease protein-protein interactions. The reduced propensity of the protein to aggregate, indicated by no increase in turbidity of the solution after storage, resulting in an increase in solubility. This increase in the solubility allowed for the enzyme to exhibit higher apparent activity. Wild-type KdcA shows substrate inhibition. Since the substrate was an acid, at higher concentrations it could destabilize the purified enzyme and induce loss of activity. The MK fusion protein shows the best activity; this is because it is fused to only one protein with no histidine tag. The HGK and MKH fusions have hexyl-histidine tags attached. In the buffer solution, the his-tag is deprotonated (weakly negatively charged) and the acid is negatively charged. This could explain why, when we add the his-tag, the activity drops slightly. Ultimately, the fusion proteins exhibited high activity in solution.

KdcA fusion protein thermal and solvent stability in free solution

The fusion proteins show significantly faster kinetics in solution versus that for wild-type (Table 2). Wild-type KdcA loses 50% of its activity after being heated for 10 min to 45°C and nearly all of its activity at 50°C (Figure 3A). The fusion proteins retain over 50% activity at 50°C and does not lose all kinetic activity until 60°C. In addition to the increase in thermal stability, the fusion proteins show stability in the presence of isobutanol. Wild-type KdcA starts to lose activity at 0.3% (v/v) isobutanol, whereas the fusion proteins do not show any loss of activity until above 1% (v/v) isobutanol (Figure 3B). The increased activity and stability analysis results with temperature and isobutanol as surrogates suggest that the fusion protein would retain some of its activity when immobilized.

The fusion proteins showed increased tolerance to heat and isobutanol over that for wild type in free solution. The presence of a coupled stable protein like GFP or MBD helped keep the enzyme soluble. As the conditions of the system become less favorable, the KdcA begins to become unstable; however, the GFP and MBD remain stable and help keep the enzyme active for longer. Without the stabilizing fused protein partner, the enzyme's instability induces aggregation. The fusion protein was not observed to keep KdcA stable under all the conditions tested; this indicates that there is a point at which the KdcA portion of the fusion loses native structure, resulting in a loss of function.

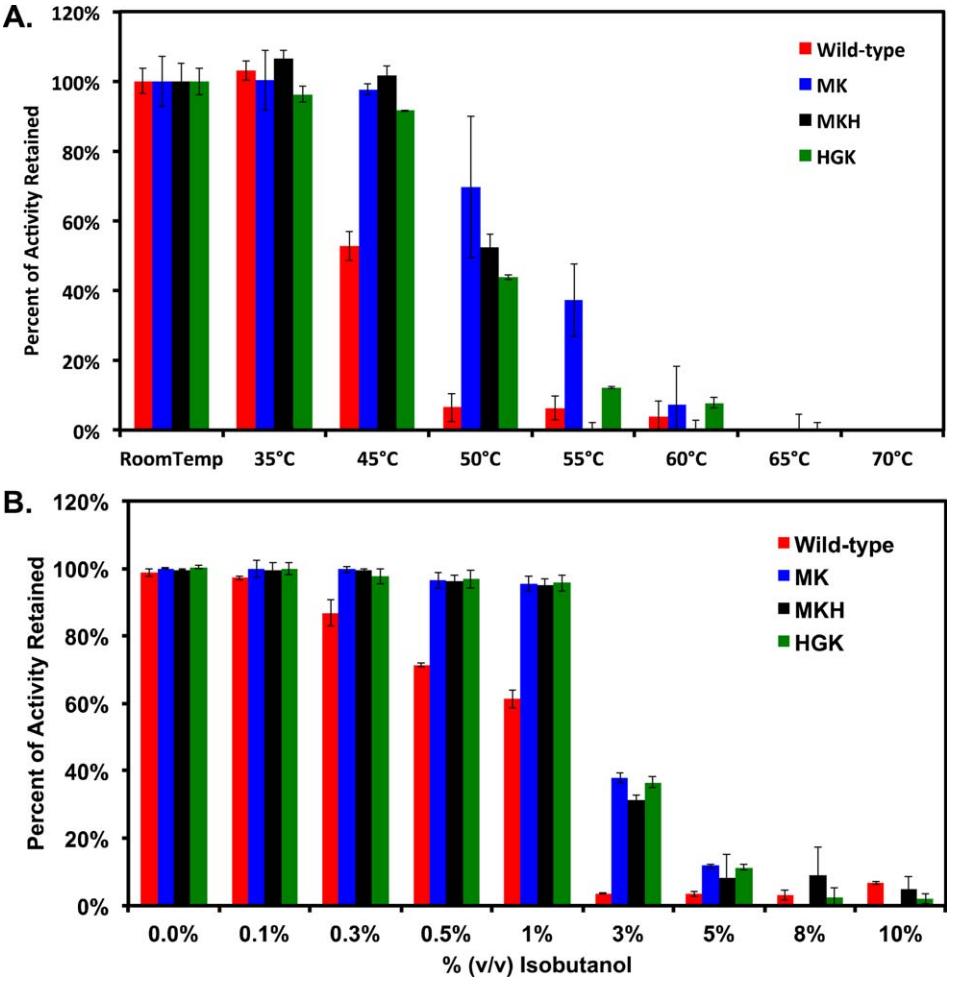

Figure 3. KdcA thermal and solvent stability.

A. Heat stability; 2 mL solution were heated to various temperatures for 10 min in glass vials. The vials were allowed to cool to room temperature in ambient conditions. Once cooled, 200 μL aliquots of the enzyme solutions were each pipetted 96-well plates and assayed. B. Isobutanol tolerance; KdcA and KdcA fusion proteins were assayed in the presence of different amounts of spiked isobutanol.

KdcA fusion protein immobilization stability

Equal amounts of each KdcA fusion protein was reacted with amino-epoxy methacrylate resin, resulting in covalently immobilized enzymes. The amounts of immobilized protein varied: 7 nmol of wild-type KdcA was immobilized on 250 mg of amino-epoxy methacrylate resin, 11 nmol of MK was immobilized on 250 mg of amino-epoxy methacrylate resin, 12.75 nmol HGK was immobilized on 250 mg of amino-epoxy methacrylate resin, and 13.25 nmol of MKH was immobilized on 250 mg of amino-epoxy methacrylate resin. Currently, the mechanism behind the varying immobilization densities of each protein is not well understood. Each immobilized enzyme sample was compared with equivalent amounts of enzyme free in solution. Figure 4 shows that the wild-type enzyme loses all activity when immobilized. All the fusion proteins retained activity when immobilized. MKH gave the best kinetics and best immobilization (53 nmol of enzyme/g of resin) (Figure 4D); therefore, we selected it to be used in our fully immobilized enzyme system.

The addition of GFP or MBD was enough to stabilize the KdcA during immobilization. Also, the MKH fusion exhibited higher activity when immobilized than the other two constructs. Lastly, the MKH fusion immobilized to a greater extent. The higher degree of immobilization leads to a greater amount of enzyme per resin particle; therefore, there were more catalytic sites in the same volume of resin. The increased immobilization amount was likely due to the fact that there is a portion of other protein on both sides of KdcA: MBD on one end, and the his-tag on the other. The immobilization reaction was between an epoxy and a free NH_2 group. The most solvent exposed residues would be the first to react. By creating more solvent exposed sections on the two sides of the enzyme, one can bias the immobilization to those sites rather than to the KdcA structure, where it could disrupt the stability of the enzyme. The MKH fusion is used below to complete the two-step immobilization reaction system

Low concentration immobilization reaction scheme

In order to assess the impact of cofactor recycling on the two-step immobilized enzyme system, we compared the ability of the reaction to go to completion in the absence and presence of NADH recycling (without and with addition of formate, respectively) (Figure 1). When limited cofactor NADH (1.2 μmol) and 64 μmol of ketoisovaleric acid were reacted with immobilized MKH, immobilized ADH, and FDH in solution, without formate, only the intermediate (isobutyraldehyde) was produced

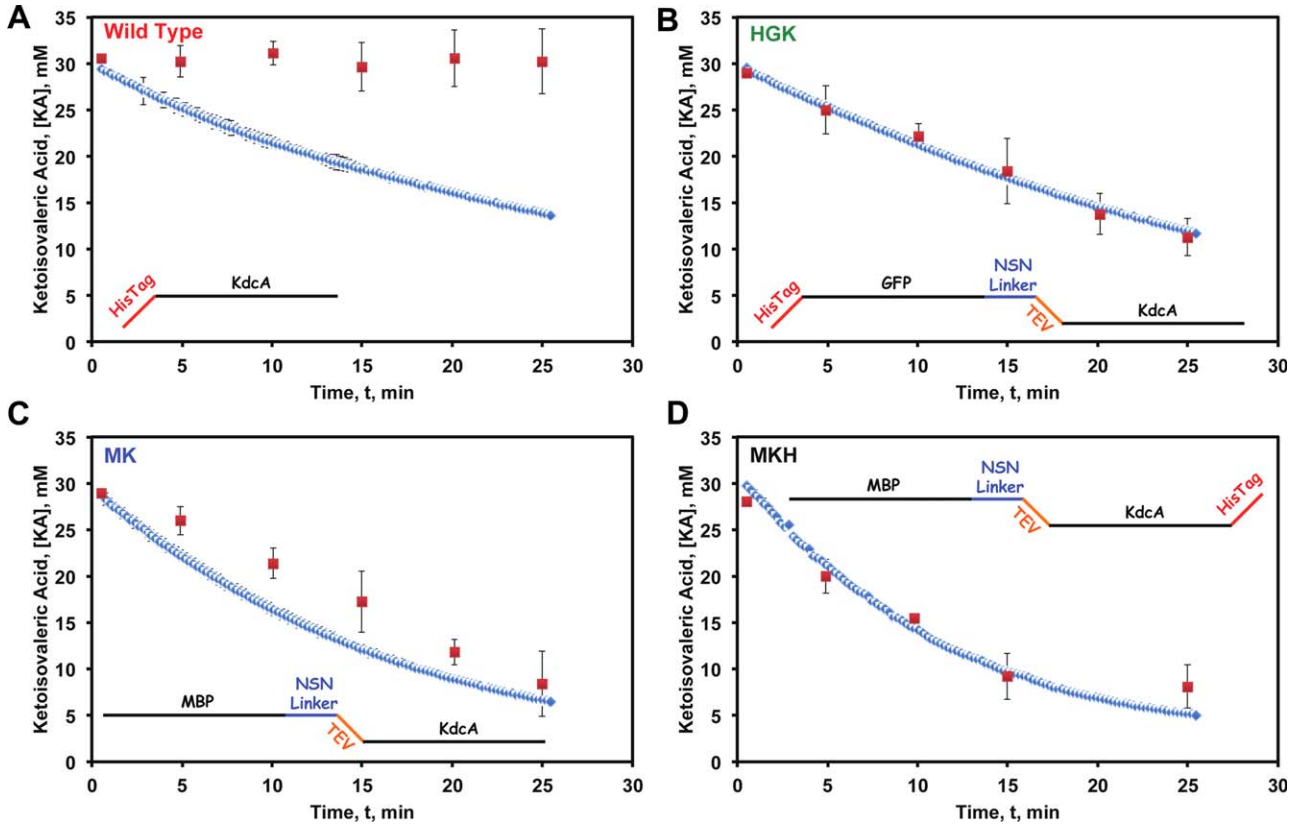

Figure 4. KdcA activity immobilized on amino-epoxy methacrylate (■) versus free in solution (◆).

A. Wild-type keto-acid decarboxylase (wild type). B. His-tag-green florescent protein keto-acid decarboxylase (HGK). C. Maltose binding domain keto-acid decarboxylase (MK), and D. Maltose binding domain keto-acid decarboxylase his-tag (MKH) enzymatic activity.

Table 3. Small-Scale Reaction with Immobilized ADH, Immobilized KdcA and FDH in Solution*

	Keto-isovaleric acid (μmol)	NADH (μmol)	Isobutyraldehyde (μmol)	Isobutanol (μmol)
Starting reaction mixture	64	1.2	–	–
Reaction w/o formate	Negligible	Negligible	62 ± 1.2	Negligible
Reaction w/formate	Negligible	0.73 ± 0.16	29 ± 2.2	35 ± 2.2

*1 mL reaction volume containing 100 mg resin with immobilized ADH, 100 mg resin with immobilized MKH, and 250 μg FDH reacted on an end-over-end mixer for 24 h at 22°C.

(Table 3). The same system reacted with 74 μmol formate and isobutanol was produced. There was a 55 ± 3.5% conversion from acid to alcohol after 24 h. From a mass balance, the cofactor was recycled about 30 times before the reaction reached steady state.

Our goal was to use a two-step immobilized enzyme system with cofactor recycling to produce isobutanol. When the cofactor (NADH) was limited in concentration, the reaction only produced the isobutyraldehyde intermediate (Table 3). Isobutanol was only produced when formate was added to regenerate the cofactor. We find that the conversion of isobutyraldehyde to isobutanol was, however, incomplete. Initially, formate concentrations were set slightly above the concentration of the starting substrate. Formate is an inexpensive sacrificial substrate; therefore, it can be added in excess. To determine why the reaction system did not proceed to completion, we increased the scale of the reaction and added the regenerating substrate (formate) in great excess. We expected the system with increased concentrations to produce more alcohol. The increased amount of formate should drive the final reaction towards the alcohol product. This is detailed below.

High concentration immobilization reaction scheme

To drive the reaction to completion, formate was added in greater excess and the reaction as a whole was scaled up to produce a larger theoretical percent of isobutanol. Ketoisovaleric acid (320 μmol) was reacted with 6 μmol of NADH and 1,500 μmol of formate. If the reaction were to go to completion, a total of 2% (v/v) of isobutanol would theoretically be produced. At 24 h the reaction reached a steady state with 57 μmol of starting material, 162 μmol of intermediate, and 101 μmol of alcohol (Table 4). These concentrations were determined via absorbance at 340 nm for the starting material, and from gas chromatography peaks for the intermediate and product. The resulting concentration of isobutanol was 0.5% (v/v); this result demonstrates that the second reaction is reversible[16,17] and the presence of a significant concentration of isobutanol and isobutyraldehyde inhibits the first reaction. By using an in situ removal technique, such as pervaporation, the reaction could be driven to completion.

The reaction system produced a final concentration of 0.5% (v/v) isobutanol. Unreacted starting material (acid) and

Table 4. High Concentration Reaction Scheme with Immobilized ADH, Immobilized KdcA, and FDH in Solution*

	Ketoisovaleric Acid (μmol)	NADH (μmol)	Isobutyraldehyde (μmol)	Isobutanol (μmol)
Starting reaction mixture	320	4	–	–
Reaction cofactor recycling	57 ± 2.1	2 ± 1.1	162 ± 1.8	101 ± 1.8

*1 mL reaction volume containing 100 mg resin with immobilized ADH, 100 mg resin with immobilized MKH, and 250 μg FDH reacted on an end-over-end mixer for 24 h at 22°C.

Table 5. Isobutanol Titers from In Vivo and In Vitro Production without in situ product removal

Host Cell	Titers (g/L Culture)	Titers (% (v/v))	Substrate	Mole Isobutanol/ Mole Substrate*	References
Saccharomyces cerevisiae	1.62	0.2	Glucose	0.038	2
Clostridium cellulolyticum	0.66	0.082	Cellulose	–	18
Saccharomyces cerevisiae	0.635	0.08	Glucose	0.016	19
In vitro	–	0.095	Glucose	0.412	7
In vitro	–	0.2 to 0.5	Ketoisovaleric acid	0.316	This work

*Max theoretical yield: 1 mol of isobutanol/mole of substrate.

intermediate (aldehyde) were still present in the solution. The first reaction involved the conversion of the acid to aldehyde (Figure 1). This reaction produced CO_2, and was nearly irreversible because of the creation of a dissolved gas product. The second reaction involved the conversion of the aldehyde to the alcohol. This reaction is a reversible reaction; in natural systems ADH is used to deprotonate an alcohol to produce an aldehyde.[17] Even with the driving force imposed by FDH, the reaction will still reach equilibrium. The reason the initial substrate was still present was likely due to the build-up of the aldehyde and alcohol, which are solvents that can harm the stability and affect the activity of the KdcA fusion protein.

Current isobutanol production titers obtained by in vivo systems amount to 1.62 g/L of culture; this concentration leads to a 0.2% (v/v) of isobutanol in the cell media (Table 5). Our novel in vitro immobilized enzyme system run at the low concentration levels described in Table 3 was able to produce 0.2% (v/v) isobutanol. By simply increasing our feed concentration fivefold, 0.5% (v/v) concentration of isobutanol was achieved. In vivo systems have production limitations because of the toxicity and inhibition of the isobutanol. Krause et al. engineered *Corynebacterium glutamicum* to over produce ketoisovaleric acid.[20] The bacteria generate 22 g of ketoisovaleric acid/L of culture. If this is the starting concentration, which is an order of magnitude higher than the isobutanol titers, the immobilized enzyme system should theoretically produce 14 g/L or 1.75% (v/v) of isobutanol. By combining higher starting concentration with in situ product removal, an order of magnitude increase in isobutanol production could possibly be reached.

In situ isobutanol removal—pervaporation

Many options for removal of *n*-butanol have been reported in the literature, but the number of options studied for isobutanol is much lower. Adsorption,[21–25] gas stripping,[26,27] and pervaporation[28,29] have all been evaluated for *n*-butanol. Below we summarize the main results from a separate paper that focused on the removal of isobutanol (6% (v/v)) from aqueous solution using pervaporation.[30] Membrane materials have a large effect on the efficiency of alcohol removal by the pervaporation (PV) process.[31] By evaluating the properties of the materials, one can determine how diffusivity and sorption properties change. Traditional PV membranes often employ an ultrathin layer of a homogeneous silicone rubber, e.g., polydimethylsiloxane (PDMS).[31] These membranes are limited to a single surface chemistry and need to rely on membrane doping[32] to achieve selectivity and separation. Our novel hydrophobic brush membrane exhibited a total flux, J, of 0.8 ± 0.15 LMH (L/m^2 h); this is comparable to the fluxes for commercial PDMS membranes Sil5 and Sil20 of $J = 0.7 \pm 0.06$ LMH and 1 ± 0.11 LMH, respectively. However, the selectivity for the brush membrane was $\alpha = 10.1 \pm 0.86$ compared with $\alpha = 6.7 \pm 0.11$ and 6.7 ± 0.05 for Sil5 and Sil20 respectively.[30,32] This new class of membrane can be utilized with our reaction system to create in situ removal of isobutanol from the system. The Liao group has employed gas stripping to remove isobutanol from an *Escherichia coli* fermentation system and obtained final titers of 0.082% (v/v).[18] We speculate that by adding PV to our production system we will be able to increase our titers from 0.5% (v/v) to 1.25% (v/v), far above of 0.2, 0.082 and 0.08% (v/v) reported for in vivo systems.[23–25] The Liao group has in other cases been able to achieve 0.675% (v/v)[34] and 6.25% (v/v)[35] when employing *in situ* removal of isobutanol.

To overcome substantial challenges with in vivo formation of isobutanol, an alternate simplified cell-free immobilized enzyme system was developed and successfully tested. This involved immobilization and characterization of two enzymes needed to convert ketoisovaleric acid to isobutyaldehyde (KdcA) and then to isobutanol (ADH). Immobilizing ADH with high activity was relatively easy. However, KdcA needed to be fused with stabilizing proteins (GFP and MBD) to retain stability and activity. Fusion proteins of KdcA exhibited increased activity and stability even when immobilized or exposed to increasing temperature or isobutanol concentrations. By coupling GFP or MBD to KdcA, the solubility of the protein also increased. This increase in solubility was largely due to a reduction of KdcA self-interactions. By making the enzyme more soluble, higher kinetic activity and increase stability was obtained. Ultimately, MKH was the easiest fusion to purify and immobilize. This fusion protein was used in conjunction with immobilized ADH to produce a two-step immobilized enzyme reaction system.

The enzyme FDH functions well in solution as a cofactor with a recycling mechanism. The reaction system with free FDH and cofactor recycling achieved a final concentration of 0.5% (v/v) isobutanol. The in vitro system did not go to completion because under limited cofactor conditions the final reaction (aldehyde to alcohol) was reversible. This production

method can be driven to completion in which isobutanol is continuously removed from the reaction vessel. In situ removal of isobutanol is critical for our system to be viable. In a separate manuscript we discuss detailed methods of removing alcohols from aqueous environments and how atmospheric plasma graft polymerization can be used to create new surface functionality on membranes. These new hydrophobic brush membranes achieve better isobutanol separations than commercial silicone membranes.

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