Supporting information of " A Bio-Catalytic Approach to Aliphatic Ketones "

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1. Cloning procedure

Reagents. Chemicals were obtained from Sigma-Aldrich or Fisher Scientific. Restriction enzymes, DNA ligation kit and Phusion DNA polymerase were from New England Biolabs. Oligonucleotides were from Eurofins MWG Operon.

Plasmids. The 4702 bp fragment of wild type *leuABCD* operon (Figure S1a) and the *leuABCD* operon carrying G462D/H97L/S139G mutations (Figure S1b) were obtained from pZE_LeuABCDKA6 plasmid (1) by Acc65I digestion, and inserted into pZAlac_ilvD_alsS (pIBA1) (2) to create pIVC1 (see Figure S2 for plasmid map) and pIVC2 (see Figure S3 for plasmid map). pIVC3-15 encodes different combinations of decarboxylase and dehydrogenase (Figure S1c).

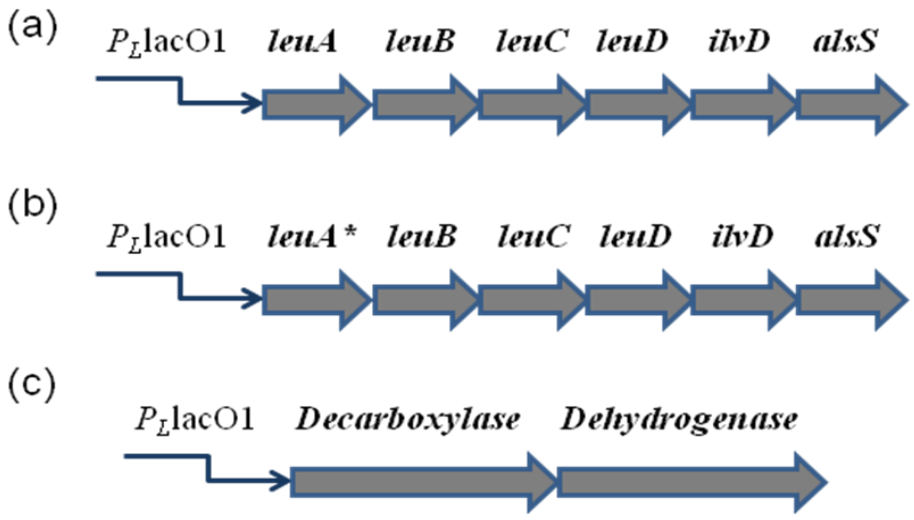


Figure S1. (a) Synthetic operon for protein overexpression to drive the carbon flux towards ketoleucine. (b) Synthetic operon for protein overexpression to drive the carbon flux towards ketohomoleucine. (c) Synthetic operon for protein overexpression for decarboxylase and dehydrogenase.

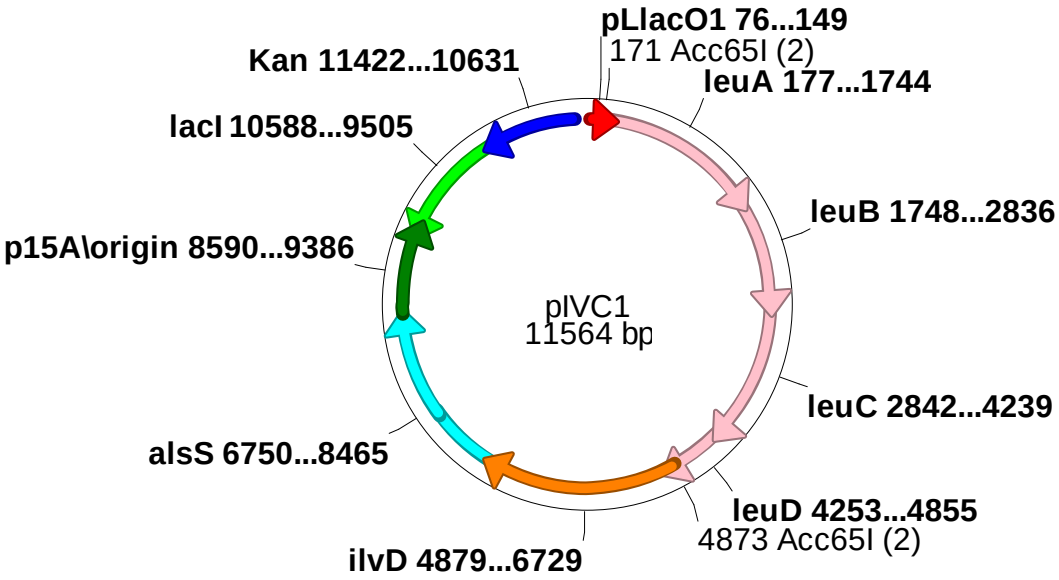


Figure S2. Plasmid map of pIVC1.

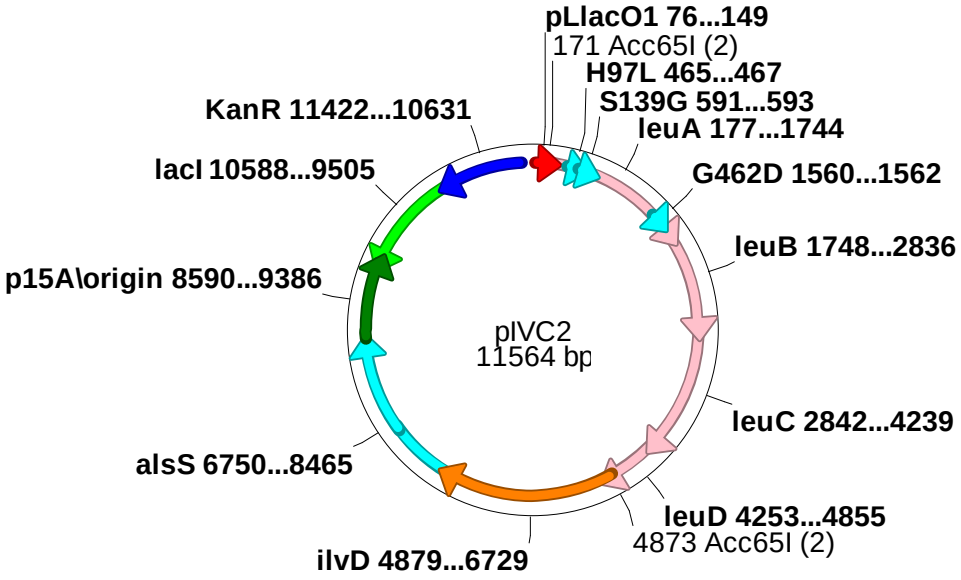


Figure S3. Plasmid map of pIVC2.

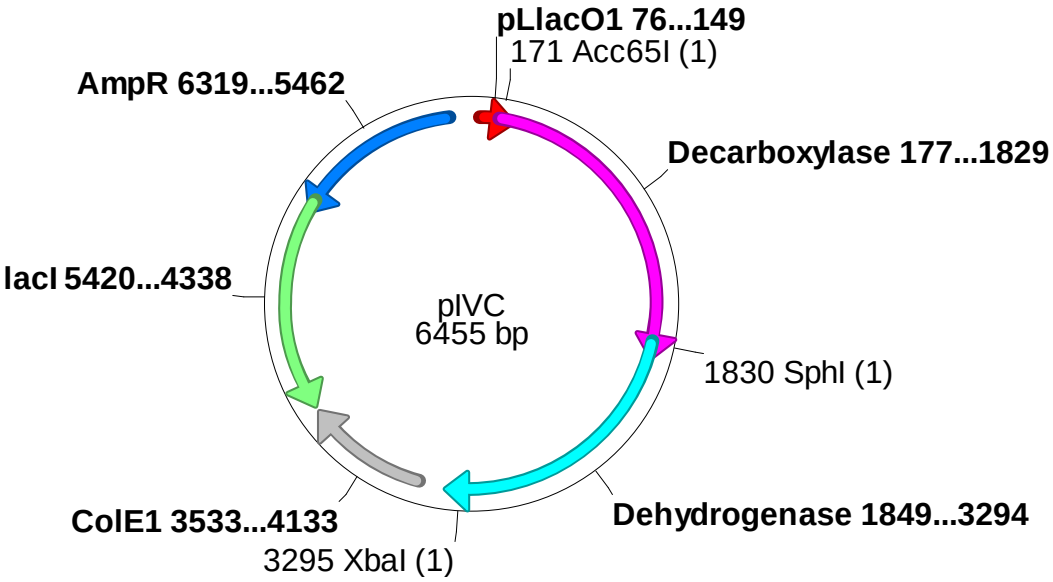


Figure S4. Plasmid map of pIVC$_{3\text{-}15}$ encoding decarboxylase and dehydrogenase

Based on the crystal structure of the KIVD active site, on top of the *V461A* mutation (1), the F381L and F382L mutation were performed with oligo pair kivd_F381Lfwd/kivd_F381Lrev or kivd_F382Lfwd/kivd_F382Lrev. The M538A and F542L mutations were obtained with primer pair kivd_accfwd/kivd_M538Arev and kivd_accfwd/kivd_F542Lrev. The gene fragments of KIVD mutants were used to replace the wild type KIVD fragment in pIBA7 plasmid (2) or in pIBA8 plasmid (2) to form pIVC3 (*V461A/ F381L*), pIVC4 (*V461A/ F382L*), pIVC5 (*V461A/ M538A*), pIVC6 (*V461A/ F542L*), or pIVC8 (*V461A/ F381L*), pIVC9 (*V461A/ F382L*), pIVC10 (*V461A/ M538A*) and pIVC11 (*V461A/ F542L*), respectively. The IPDC gene fragment was amplified from the genomic DNA of *Salmonella typhimurium* with primers IPDC_accfwd and IPDC_sphrev, digested with Acc65I and SphI, and then inserted into the corresponding restriction sites of pIBA7 or pIBA8 to form plasmid pIVC7 or pIVC12, respectively. According to the alignment results between KIVD and IPDC, the V465A mutation on IPDC was obtained using primers IPDC_V465Afwd and IPDC_V465Arev. The V540A and L544A mutations were obtained using primer pair IPDC_accfwd/IPDC_V540Arev and IPDC_accfwd/IPDC_L544Arev. The digested fragments of V465A, V540A and L544A IPDC mutants were used to replace the wild type IPDC fragment in the plasmid pIVC12 to form pIVC13 (*V465A*), pIVC14 (*V540A*), and pIVC15 (*L544A*).

Table S1. Oligonucleotides for cloning.

Name	sequence
kivd_F381Lfwd	GTTGCTGAACAAGGGACATCACTGTTTGGCGCTTCATCAATTTTC
kivd_F381Lrev	GAAAATTGATGAAGCGCCAAACAGTGATGTCCCTTGTTCAGCAAC
kivd_F382Lfwd	GCTGAACAAGGGACATCATTCCTGGGCGCTTCATCAATTTTCTTA
kivd_F382Lrev	TAAGAAAATTGATGAAGCGCCCAGGAATGATGTCCCTTGTTCAGC
kivd_M538Arev	GGGCCCGCATGCTTATGATTTATTTTGTTCAGCAAATAGTTTGCCTGCTTTTTTCAGTA
kivd_F542Lrev	GGGCCCGCATGCTTATGATTTATTTTGTTCAGCCAGTAGTTTGCCCATTTTTTTCAGTA
IPDC_accfwd	GGGCCC GGTACC ATGCAAAACCCCTATACCGTGGCCGA
IPDC_sphrev	GGGCCC GCATGC TTATCCCCCGTTGCGGGCTTCCAGCG
IPDC_V465Afwd	GCTGCTCAACAATGACGGCTATACCGCTGAGCGCGCCATTCACGGCGCGGCCCAGCGGT
IPDC_V465Arev	ACCGCTGGGCCGCGCCGTGAATGGCGCGCTCAGCGGTATAGCCGTCATTGTTGAGCAGC
IPDC_V540Arev	GGGCCCGCATGCTTATCCCCCGTTGCGGGCTTCCAGCGCCCGGGTCGCGGTACGCAGTA
IPDC_L544Arev	GGGCCC GCATGCTTATCCCCCGTTGCGGGCTTCCGCCGCCCGGGTCACGGTACGCAGTA
padA_bamfwd	GACTAT GGATCC ATGACAGAGCCGCATGTAGCAGT
padA_bamrev	GACTAT GGATCC TTAATACCGTACACACACCGACTTAGTT
IPDC_bamfwd	GGGCCC GGATCC ATGCAAAACCCCTATACCGTGGCCGA
IPDC_bamrev	GGGCCC GGATCC TTATCCCCCGTTGCGGGCTTCCAGCG
kdh_{ba}_bamfwd	GGGCCC GGATCC ATGGCTAACGTGACTTATACGGATAC
kdh_{ba}_bamrev	GGGCCC GGATCC TTAGACCGCCATCACCGTCACCGACT

* The underlines in this Table means the mutation site or restriction site for cloning.

2. Knocking out Chromosomal Genes

Gene knockout was performed by P1 phage transduction and the strains to get the phage were obtained from the Keio collection (3). Colonies containing the correct deletions were verified by PCR. PCP20 plasmid was then used to remove the kanamycin resistance marker. A deletion strain AKO5 based *E. coli* K-12 strain BW25113 (Δ*pta*, Δ*poxB*, Δ*adhE*, Δ*ldhA*, Δ*yqhD*) was obtained for the bioreactor fermentation.

3. Collection of homoketoleucine substrate

The strain carrying only pIVC2 plasmid was subject to fermention in 125 mL flasks for 48 h. The fermentation product of homoketoleucine was collected by using the fraction collector of an Agilent 1260 Infinity HPLC. The pH of homoketoleucine collect was then adjusted to 2.0, and ethyl acetate was added to extract homoketoleucine. The top organic layer carrying homoketoleucine was separated from the aqueous solution, and dried in a speed vacuum.

4. Enzymatic assay

Protein Expression and Purification. The gene fragments of KDH_{ba} and the wild type IPDC were PCR amplified from the pIVC12 plasmid template with primer pairs kdh_{ba}_bamfwd/kdh_{ba}_bamrev and IPDC_bamfwd/IPDC_bamrev, respectively. The gene fragment of *L544A* IPDC mutant was PCR amplified from the pIVC15 plasmid template with primers IPDC_bamfwd and IPDC_bamrev. After digestion with BamHI, the three gene fragments were inserted into expression plasmid pQE9 (Qiagen) to yield pQE9-KDH_{ba}, pQE9_IPDC and pQE9-IPDC*L544A*. These plasmids were transformed into *E. coli* strain BL21. 2mL overnight pre-cultured BL21 cells were inoculated in 200 ml 2XYT rich medium containing 50 mg/L ampicillin and 25 mg/L kanamycin. 0.1 mM IPTG was added into the medium to induce the expression of recombinant proteins. Cell pellets were lysed by sonication in a buffer containing 250 mM NaCl, 2 mM DTT, 5 mM imidazole and 50 mM Tris pH 9. Enzymes were purifed by passing cell lysates through Ni-NTA columns with the application of a stepwise gradient of imidazole (up to 250 mM). Purifed enzyme solutions were buffer exchanged with the Amicon Ultra centrifugal filters (Millipore): KDH_{ba} was exchanged into buffer 1 (50 μM tris buffer, pH 8.0, 1 mM MgSO4, and 20% glycerol); wild type IPDC and L544A IPDC to buffer 2 (50 μM tris buffer, pH 8.0, 1 mM MgSO4, 0.2 mM ThDP, and 20% glycerol). These solutions were flash frozen and stored at -80 ^{o}C.

Chracterization of PadA, KDH_{ba}, wild type IPDC and IPDC of L544A. Protein concentration was measured by UV absorbance at 280 nm. For characterization of PadA and KDH_{ba}, the assay buffer (50 mM NaH2PO4, pH 8.0, 1mM DTT) contained 0.5 mM NAD^+ and 0.2-4 mM substrates. Enzymatic reactions were initiated by adding 25 nM PadA or KDH_{ba}. The reactions were monitored by measuring the UV absorbance at 340 nm (extinction coefficient, 6.22 mM^{-1} cm^{-1}) as a consequence of NADH generation.

The decarboxylation activities of wild type IPDC and *L544A* IPDC were measured using a coupled enzymatic assay method. Excess PadA or KDH_{ba} was included in the reaction mixture to immediately oxidize the aldehyde products into the corresponding acids, and concomitantly, the cofactor NAD^+ was reduced to NADH. The assay mixture contained 0.5 mM NAD^+, 0.1 μM PadA or KDH_{ba} and 0.2-4 mM 2-keto acids in the reaction buffer (50 mM NaH2PO4, pH 6.8, 1 mM $MgSO_4$, 0.5 mM ThDP) with a total volume of 80 μl. The reaction was started by the addition of 25 nM wild type IPDC or L544A IPDC. The reaction progress was monitored by determining the generation of NADH (absorbance at 340 nm). Kinetic parameters (k_{cat} and K_m) were determined by fitting initial velocity to the Michaelis–Menten equation using Origin software.

4. Fermentation procedure

Strains. The *E. coli* host was transformed with the pIVC1 plasmid and another plasmid from pIBA4 to pIBA8 or from pIVC3 to pIVC7 for isovalerate production. To produce isocaproate, the *E.coli* host was transformed with pIVC2 plasmid and another plasmid from pIBA4 to pIBA8 or from pIVC8 to pIVC15.

Shake flask fermentation. Overnight culture was diluted 25 fold into 5 ml M9 medium (plus 0.5% yeast extract and 4% glucose) in a 125-ml conical flask. Ampicillin (100 mg/L) and kanamycin (25 mg/L) were added to maintain the transformed plasmids. The culture medium was buffered with $CaCO_3$ powder. isopropyl-beta-D-thiogalactoside (IPTG) was added at a concentration of 0.1 mM to induce protein expression. The fermentation flasks were kept in a 30 ℃ shaker (250 rpm) for 48 hours.

Product distribution in shake flask fermentation. The host strain is AKO1(BW25113, Δ*yqhD*).

Table S2. Major products of isovalerate fermentation in shake flask.

Strain	Titer (g/L)			
	isobutyrate	isobutanol	3-methyl-butanol	isovalerate
KIVD + AldB	0.36±0.007	0.24±0.046	4.47±0.046	**0.76±0.164**
KIVD + AldH	0.37±0.007	0.28±0.039	0.37±0.028	**5.78±0.530**
KIVD + KDHba	0.42±0.054	0.17±0.017	2.10±0.034	**7.11±0.013**
KIVD + PadA	0.74±0.075	0.11±0.021	1.63±0.082	**7.53±0.064**
KIVD *(V461A/ F381L)* + PadA	0.10±0.010	0.04±0.028	1.63±0.066	**3.56±0.069**
KIVD *(V461A/ F382L)* + PadA	0.16±0.014	0.02±0.000	0.66±0.017	**0.51±0.016**
KIVD *(V461A/ M538A)* + PadA	0.12±0.011	0.03±0.010	1.45±0.093	**3.21±0.204**
KIVD *(V461A/ F542L)* + PadA	0.07±0.006	0.03±0.005	2.03±0.061	**4.30±0.326**
IPDC + PadA	0.51±0.024	0.19±0.005	1.19±0.100	**8.91±0.278**

Table S3. Major products of isocaproate fermentation in shake flask.

Strain	Titer (g/L)					
	isobutyrate	isovalerate	isobutanol	3-methyl -butanol	4-methyl-pentanol	**isocaproate**
WT KIVD + AldB	0.74±0.094	0.17±0.006	0.32±0.068	1.21±0.074	1.07±0.058	**0.24±0.006**
WT KIVD + AldH	3.72±0.591	2.51±0.581	0.16±0.042	0.46±0.255	0.23±0.071	**1.30±0.398**
WT KIVD + KDHba	1.26±0.074	1.56±0.135	0.64±0.102	0.87±0.058	0.04±0.012	**2.69±0.066**
WT KIVD + PadA	1.78±0.079	1.80±0.119	0.06±0.005	0.74±0.025	0.61±0.104	**1.88±0.089**
KIVD *(V461A/ F381L)* + KDHba	0.28±0.061	0.02±0	0.02±0	0.12±0	0.01±0.005	**1.58±0.082**
KIVD *(V461A/ F382L)* + KDHba	0.13±0.01	0±0	0.02±0.005	0.02±0.005	0.02±0.008	**0.66±0.011**
KIVD *(V461A/ M538A)* + KDHba	0.26±0.067	0.04±0.006	0.02±0	0.11±0.006	0.02±0.005	**2.76±0.032**
KIVD *(V461A/ F542L)* + KDHba	0.23±0.014	0.05±0	0.06±0.01	0.19±0.005	0.02±0.01	**3.30±0.027**
WT IPDC + KDHba	0.77±0.014	2.48±0.029	0.71±0.025	0.74±0.036	0.02±0.005	**2.67±0.070**
IPDC(V465A) + KDHba	0.06±0.006	0.19±0.029	0.18±0.021	0.39±0.016	0.01±0.005	**4.14±0.189**
IPDC(V540A) + KDHba	0.22±0.028	0.29±0.029	0.14±0.01	0.70±0.037	0.09±0.012	**4.35±0.056**
IPDC(L544A) + KDHba	0.21±0.058	0.20±0.109	0.09±0.039	0.55±0.101	0.20±0.095	**5.02±0.014**

Bioreactor fermentation. Seeding medium has the following composition (g/L): glucose, 10; $(NH_4)_2SO_4$, 1.8; K_2HPO_4, 8.76; KH_2PO_4, 2.4; sodium citrate, 1.32; yeast extract, 15; ampicillin, 0.1; kanamycin, 0.05. Fermentation media for bioreactor cultures contained the following components (g/L): glucose, 30; $(NH_4)_2SO_4$, 3; K_2HPO_4, 14.6; KH_2PO_4, 4; sodium citrate, 2.2; yeast extract, 25; $MgSO_4.7H_2O$, 1.25; $CaCl_2.2H_2O$, 0.015, calcium pantothenate, 0.001; Thiamine, 0.01; ampicillin, 0.1; kanamycin, 0.05; and 1 mL/L of trace metal solution. Trace metal solution contained (g/L): NaCl, 5; $ZnSO_4.7H_2O$, 1; $MnCl_2.4H_2O$, 4; $CuSO_4.5H_2O$, 0.4; H_3BO_3, 0.575; Na2MoO4.2H_2O, 0.5; $FeCl_3.6H_2O$, 4.75; 6N H_2SO_4, 12.5 mL. The feeding solution contained (g/L): glucose, 600; $(NH_4)_2SO_4$, 5; $MgSO_4.7H_2O$, 1.25; yeast extract, 5; $CaCl_2.2H_2O$, 0.015; calcium pantothenate, 0.001; Thiamine, 0.01; ampicillin, 0.1; kanamycin, 0.05, 0.2 mM IPTG; and 1 mL/L of trace elements.

Cultures of *E. coli* were performed in a 1.3 L Bioflo 115 fermentor (NBS, Edison, NJ USA) using a working volume of 0.6 L. The fermentor was inoculated with 10% of overnight pre-culture with seeding medium and then the cells were grown at 37 ℃, 30% dissolved oxygen (DO), and pH 7.0. When OD_{600} was 8.0, 0.2 mM IPTG was added and the temperature was decreased to 30 ℃ to start isovalerate production. The pH was controlled at 7.0 by automatic addition of 200 g/L $Ca(OH)_2$ slurry. Air flow rate was maintained at 1 vvm in the whole process. Dissolved oxygen (DO) was maintained about 10% with respect to air saturation by raising stiring speed (from 300 to 800 rpm). The glucose level in the fermentor was kept about 10 g/L by inputting feeding medium continuously. Fermentation samples were collected to determinate concentrations of isovalerate, organic acids and glucose.

5. Catalyst preparation and characterization

Supported CeO_2 catalysts were prepared by incipient wetness impregnation of the dried support with an aqueous solution of commercial $Ce(NO_3)_3$ $6H_2O$. The mixture was dried at 388K for 12h, subsequently calcined at 833K for 4h to get the final catalyst. The data for CeO_2 percentage in the catalysts were calculated theoretically. All the reagents were purchased and directly used without further purification.

The specific surface area of the samples was calculated by the BET method using a nitrogen adsorption isotherm at temperature of 77K. X-ray diffraction (XRD) experiments were performed using a Philips X'Pert XRD with Cu Kαradiation. For elemental analysis, catalysts were dissolved in concentrated nitric acid and then diluted to a Ce content of ca. 100ppm. The solutions were analyzed using an Optima 7300 DV ICP to give the actual Ce content of the catalysts. Supplementary Table S4 presents the data of surface area and Ce content of each catalyst. The XRD patterns of 10%CeO_2/TiO_2, 18% CeO_2/TiO_2, 25% CeO_2/TiO_2 and pure TiO_2 were shown in supplementary Fig. S4.

Table S4 presents the data of surface area and Ce content of each catalyst. The BET results, for CeO_2/Al_2O_3, CeO_2/ZrO_2 and CeO_2/TiO_2, indicate that the surface area of 18% CeO_2/Al_2O_3 is much larger than the other two catalysts. The surface area of 18%CeO_2/ZrO_2 is similar to that of 18% CeO_2/TiO_2. The ICP-MS results indicate that the actual content of CeO_2 in the catalysts is less than the content theoretically calculated, probably due to the loss in the preparation step.

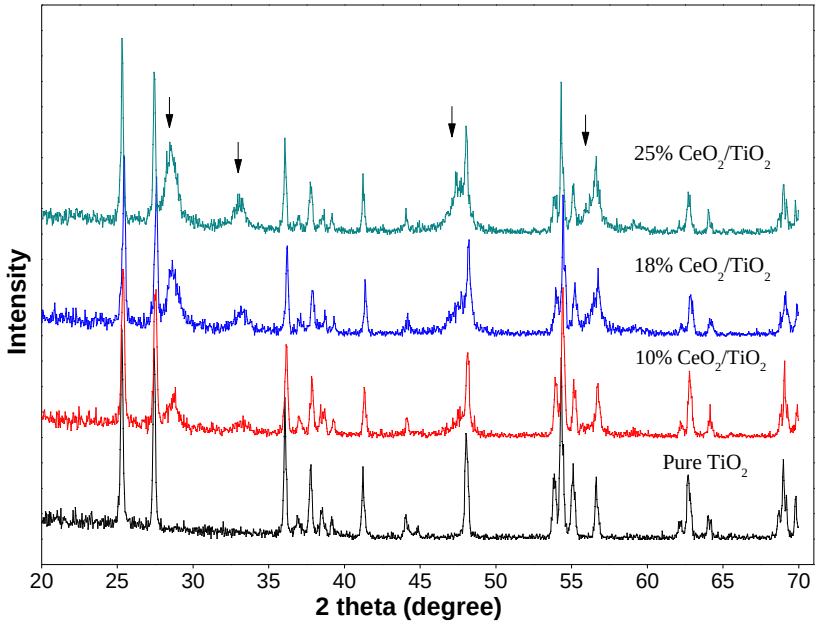
Table S4. Surface area and ICP-MS results for catalysts[a]

Catalyst	Surface area [m^2/g]	ICP-MS composition, CeO_2 [wt.%]
18% CeO_2/Al_2O_3	101.6726	15.96
18% CeO_2/ZrO_2	17.7618	16.72
10% CeO_2/TiO_2	7.1197	9.86
18% CeO_2/TiO_2	7.0765	16.83
25% CeO_2/TiO_2	10.8173	23.02

[a] Metal loadings (wt%) were referred to nominal values of CeO_2 loading on the support.

The XRD patterns of 10%CeO_2/TiO_2, 18% CeO_2/TiO_2, 25% CeO_2/TiO_2 and pure TiO_2 were shown in Fig. S5. Compared to the pattern of pure TiO_2, there were broad peaks which included the CeO_2 reference peaks at 28.6, 33.1, 47.5 and 56.4 degrees and the intensity increased with the increasing loading of CeO_2 in the catalysts, which indicate that the CeO_2 was supported by the TiO_2 lattice in the prepared catalysts.

Figure S5. XRD patterns for CeO_2/TiO_2 catalysts and pure TiO_2



6. Ketonization reactions

The reactor used for the reaction studies (Fig. S6) was a fixed-bed, downflow reactor consisting of a quartz tube (10mm i.d.) containing the catalyst bed between two plugs of quartz wool. 1g catalyst was mixed with 2g quartz granules before being filled into the quartz tube. The reactor was heated externally with a furnace. Temperature was measured using a K-type thermocouple attached to the middle of the catalyst bed. A rotameter was used to control the nitrogen flowrate. A injection pump was used to introduce the liquid feed into the reactor. The effluent product was condensed and collected in a receiver cooled by an ice trap. Before the reaction, the temperature of the fixed-bed reactor was held for 30 min, and then the feed was pumped into the reactor at an LHSV of 1 $cm^3\ g_{catalyst}^{-1}\ h^{-1}$, and high purity nitrogen was used as the carrier gas at a flowrate of $20cm^3\ min^{-1}$. In the preparation of MIBK and MIAK, all the feeds contained 40wt% water. In the preparation of DIBK, pure IVA was pumped directly into the reactor. The liquid product were analyzed using a Shimadzu GC-2014 gas chromatography with an FID detector and DM-Wax capillary column.

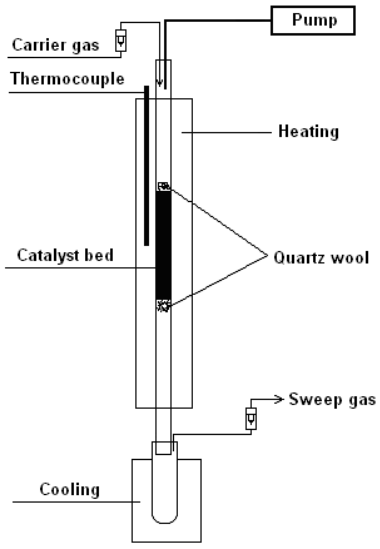


Figure S6. **Designed reactor for the study of the gas-phase ketonization reaction.**

7. References

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