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Electronic Supplementary Information

An Artificial Enzymatic Reaction Cascade for a Cell-free Bio-system Based on Glycerol

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Materials and Methods

Chemicals and materials.

Isopropyl-β-D-1-thiogalactopyranoside (IPTG), phenylmethanesulfonyl fluoride (PMSF), and dithiothreitol (DTT) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). PCR primers were obtained from Sangon (Shanghai, China). Ampicillin was purchased from Amresco (USA). Restriction enzymes were obtained from Fermentas (Lithuania). T_4 DNA ligase and fastPfu DNA polymerase were purchased from MBI (USA) and Transgen Biotech (China), respectively. The catalase in *Aspergillus niger* was purchased from Sigma-Aldrich (C3515). All other chemicals were of analytical grade and commercially available.

Strains, primers, plasmids and medium.

Bacterial strains, primers and plasmids used in this study are listed in Table S1. *Escherichia coli* BL21 (DE3) was used for protein expression, and *E. coli* DH5α was used for general cloning. Luria-Bertani (LB) medium was used for *E. coli* and *Bacillus licheniformis* 10-1-A culture and recombinant protein expression. Ampicillin was used at a concentration of 100 μg mL^{-1}.

Vector construction.

Full-length genes of *Aldo* in *Streptomyces coelicolor* A3 (with optimized *E. coli* codon usage) and *Dhad* in *Sulfolobus solfataricus* were synthesized by Sangon (Shanghai, China) and delivered in the company's standard plasmids. The genes *Aldo* and *Dhad*

were amplified using the Sangon vectors as templates and the corresponding oligonucleotides (Table S2). Genomic DNA of *B. licheniformis* 10-1-A was extracted through the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The genes *Als* and *Aldc* were amplified from genomic DNA of *B. licheniformis* 10-1-A using the corresponding oligonucleotides (Table S2). The PCR products were ligated to the pEasy-Blunt vector, and the resulting plasmids were then digested with restriction enzymes indicated in Table S2.The gel purified fragments were then ligated to the pETDuet-1 vector that had been digested with the same restriction enzymes to construct the plasmids for the expression of Aldo, DHAD, ALS and ALDC.

Recombinant proteins expression and purification.

The protein expression plasmids were introduced separately in *E. coli* BL21 (DE3). *E. coli* BL21(DE3) carrying plasmid pETDuet-*Aldo*, pETDuet-*Dhad*, pETDuet-*Als* or pETDuet-*Aldc* were grown at 30°C in LB medium with 100 μg mL^{-1} ampicillin to an optical density of 0.5 at 620 nm. Then, 1 mM IPTG was added to induce the expression of Aldo, DHAD, ALS and ALDC. After cultivation for another 12 h at 16°C, cells were harvested and washed with 0.85% (wt/vol) sodium chloride solution by centrifugation at 8,000 rpm for 5 min. The cell pellet was subsequently suspended in the binding buffer (pH 7.4; 20 mM sodium phosphate, 20 mM imidazole, and 500 mM sodium chloride) containing 0.5 mM PMSF, 1 mM DTT and 10% glycerol. Cells were disrupted by sonication (Sonics500 W; 20 KHz) for 10 min in an ice bath, and

the cell lysate was centrifuged at 8,000 rpm for 30 min at 4°C. The supernatant was loaded onto a HisTrap HP column (5 mL) and eluted with 40% binding buffer and 60% elution buffer (pH 7.4; 20 mM sodium phosphate, 500 mM imidazole, and 500 mM sodium chloride) at a flow rate of 5 mL min^{-1}. The fractions containing Aldo, DHAD, ALS and ALDC were concentrated by ultrafiltration, desalted with gel G-25 and detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme activity assays.

Aldo activity: The Aldo activity was assayed in a reaction mixture containing 100 mM HEPES buffer (pH 7.4), and 50 mM glycerol. After the conversion at 50°C for 3 h, the oxidization of glycerol to glycerate was determined via high-performance liquid chromatography (HPLC) as described below.

DHAD activity: The DHAD activity was measured in a reaction mixture containing DHAD, 20mM glycerate and 100 mM HEPES buffer (pH 7.4). The reaction mixture was incubated at 50°C for 12 h. The concentration of pyruvate produced from glycerate was determined via HPLC as described below.

Catalase activity: The activity of catalase was determined in a reaction mixture containing 20 mM hydrogen peroxide and 100 mM HEPES buffer (pH 7.4) using an Ultrospec^{TM} 2100 pro UV/visible spectrophotometer. The catalase activity was assayed by measuring the change in absorbance at 240nm corresponding to the decomposition of hydrogen peroxide (ε_{240}= 43.6 $M^{-1}cm^{-1}$).

ALS activity: The ALS activity was measured by monitoring the conversion of pyruvate to α-acetolactate in a reaction mixture containing 0.2 mM thiamine diphosphate, 10 mM pyruvate and 100 mM HEPES buffer (pH 7.4). [1]

ALDC activity: The ALDC activity was assayed by detecting the production of (3*R*)-acetoin from α-acetolactate in a reaction mixture containing 5 mM α-acetolactate and 100 mM HEPES buffer (pH 7.4).[2] α-Acetolactate was prepared immediately before use from ethyl 2-acetoxy-2-methyl-acetoacetate (Sigma-Aldrich), which could be transformed to α-acetolactate by adding two equivalents of NaOH.

Analytical methods.

Cell growth was measured via optical density measured at 620nm (OD620) using an Ultrospec^{TM} 2100 pro UV/visible spectrophotometer. The protein concentration was determined by the Lowry procedure using bovine serum albumin as the standard. [3]

Aqueous concentrations of glycerol, glyceric acid, glyceraldehydes, (3*R*)-acetoin and pyruvic acid were measured by a HPLC system (Agilent 1100 series, Hewlett-Packard), equipped with a refractive index detector. [4] The HPLC was fitted with a Bio-Rad Aminex HPX-87 H column. The mobile phase consisted of 10 mM H_2SO_4 pumped at 0.4 mL min^{-1} (55 °C).The ratio of the (3*R*)-acetoin and (3*S*)-acetoin was analyzed by a gas chromatography (GC) system (Agilent GC6820) using a fused silica capillary column (Supelco Beta DEXTM 120, inside diameter, 0.25 mm; length, 30 m). The operating conditions were as follows: nitrogen was used as the carrier gas; the injector temperature and detector temperature were both 280°C; the column oven

was maintained at 40°C for 3 min and then programmed to increase to 80°C at a rate of 1.5°C min^{-1}; the temperature was then raised to 86°C at a rate of 0.5°C min^{-1} and finally to 200°C at a rate of 30°C min^{-1}; and the injection volume was 1 μL. [5]

Full-length of *Aldo* with optimized *E. coli* codon usage.

ATGAGCGACATCACCGTTACCAACTGGGCCGGCAACATCACCTACACCGCG

AAGGAACTGCTGCGTCCGCACTCCCTGGACGCGCTGCGTGCCCTGGTGGC

GGACAGCGCCCGTGTGCGTGTGCTGGGCAGCGGTCACTCCTTCAACGAGA

TCGCCGAGCCGGGCGACGGTGGTGTTCTGCTGTCTCTGGCGGGCCTGCCGT

CCGTGGTGGACGTGGACACCGCGGCCCGTACCGTGCGTGTTGGCGGCGGT

GTGCGTTACGCGGAGCTGGCCCGTGTGGTGCACGCGCGTGGCCTGGCGCT

GCCGAACATGGCCTCTCTGCCGCACATCTCTGTTGCCGGTTCTGTGGCCAC

CGGCACCCACGGTTCTGGTATGGGCAACGGTTCTCTGGCCTCTATGGTGCG

CGAGGTGGAGCTGGTTACCGCGGACGGTTCTACCGTGGTGATCGCGCGTG

GCGACGAGCGTTTCGGCGGTGCGGTGACCTCTCTGGGCGCGCTGGGCGTG

GTGACCTCTCTGACCCTGGACCTGGAGCCGGCGTACGAGATGGAACAGCA

CGTTTTCACCGAGCTGCCGCTGGCCGGTCTGGACCCGGCGACCTTCGAGA

CCGTGATGGCGGCGGCGTACAGCGTGTCTCTGTTCACCGACTGGCGTGCGC

CGGGTTTCCGTCAGGTGTGGCTGAAGCGTCGCACCGACCGTCCGCTGGAC

GGTTTCCCGTACGCGGCCCCGGCCACCGAGAAGATGCATCCGGTGCCGGG

CATGCCGGCGGTGAACTGCACCGAGCAGTTCGGTGTGCCGGGTCCGTGGC

ACGAGCGTCTGCCGCACTTCCGCGCGGAGTTCACCCCGAGCAGCGGTGCC

GAGCTGCAGTCTGAGTACCTGATGCCGCGTGAGCACGCCCTGGCCGCCCT

GCACGCGATGGACGCGATCCGTGAGACCCTGGCGCCGGTGCTGCAGACCT

GCGAGATCCGCACCGTTGCCGCCGACGCGCAGTGGCTGAGCCCGGCGTAC

GGTCGTGACACCGTGGCCGCGCACTTCACCTGGGTTGAGGACACCGCGGC

GGTGCTGCCGGTGGTGCGTCGTCTGGAGGAGGCGCTGGTTCCGTTCGCGG

CCCGTCCGCACTGGGGTAAGGTGTTCACCGTTCCGGCGGGCGAGCTGCGT

GCGCTGTACCCGCGTCTGGCCGACTTCGGTGCGCTGGCCCGTGCGCTGGAC

CCGGCGGGTAAGTTCACCAACGCGTTCGTGCGCGGTGTGCTGGCGGGCTA

A

Table S1 Strains and plasmids used in this study.

Strain, plasmid and primer	Genotype, properties and sequence	Source or reference
Strain		
B. licheniformis 10-1-A	Wild type	6, 7
E. coli DH5α	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Novagen
E. coli BL21 (DE3)	*F⁻ ompT hsdSB (rB⁻mB⁻) gal (λ c I 857 ind1 Sam7 nin5 lacUV5 T7gene1) dcm* (DE3)	Novagen
Plasmid		
pEasy-Blunt	Cloning vector; Ap^r	Transgene
pETDuet-1	Overexpression vector; Ap^r	Novagen
pETDuet-*Aldo*	pETDuet-1 with *Aldo* gene of *S. coelicolor* A3	This study
pETDuet-*Dhad*	pETDuet-1 with *Dhad* gene of *S. solfataricus*	This study
pETDuet-*Als*	pETDuet-1 with *Als* gene of *B. licheniformis* 10-1-A	This study
pETDuet-*Aldc*	pETDuet-1 with *Aldc* gene of *B. licheniformis* 10-1-A	This study

pEasy-Blunt-*Aldo*	pEasy-Blunt with *Aldo* gene of *S. coelicolor* A3	This study
pEasy-Blunt-*Dhad*	pEasy-Blunt with *Dhad* gene of *S. solfataricus*	This study
pEasy-Blunt-*Als*	pEasy-Blunt with *Als* gene of *B. licheniformis* 10-1-A	This study
pEasy-Blunt-*Aldc*	pEasy-Blunt with *Aldc* gene of *B. licheniformis* 10-1-A	This study

Table S2 Primers used in this study.

Primer[a]	Oligonucleotide sequence (5' – 3')[b]	Gene amplified
Aldo_for	GGATCCGATGAGCGACATCACCGTTACC (BamHI)	*Aldo* in *S. coelicolor* A3
Aldo_rev	GTCGACTTAGCCCGCCAGCACACC (SalI)	*Aldo* in *S. coelicolor* A3
Dhad_ for	GGATCCGATGCCAGCAAAATTAAATAGTCCCT (BamHI)	*Dhad* in *S. solfataricus*
Dhad_ rev	GTCGACTTAAGCTGGTCTAGTCACAGCCC (SalI)	*Dhad* in *S. solfataricus*
Als_for	GAGGATCCAATGAATAATGTAGCCGCTAAAA (BamHI)	*Als* in *B. licheniformis*10-1-A
Als_rev	GTCTCGAGTCAAGATTGCTTAGAGGCT (XhoI)	*Als* in *B. licheniformis* 10-1-A
Aldc_for	GAGGATCCAATGAAAAGTGCAAGCAAACAAAA (BamHI)	*Aldc* in *B. licheniformis* 10-1-A
Aldc_rev	GTAAGCTTTTACTCGGGATTGCCTTCGG (HindIII)	*Aldc* in *B. licheniformis* 10-1-A

[a] "_for" in the primer name means that this is the sense primer; "_rev" in the primer name means that this is the antisense primer.

[b]Restriction sites are underlined, and the restriction enzymes are indicated in parentheses.

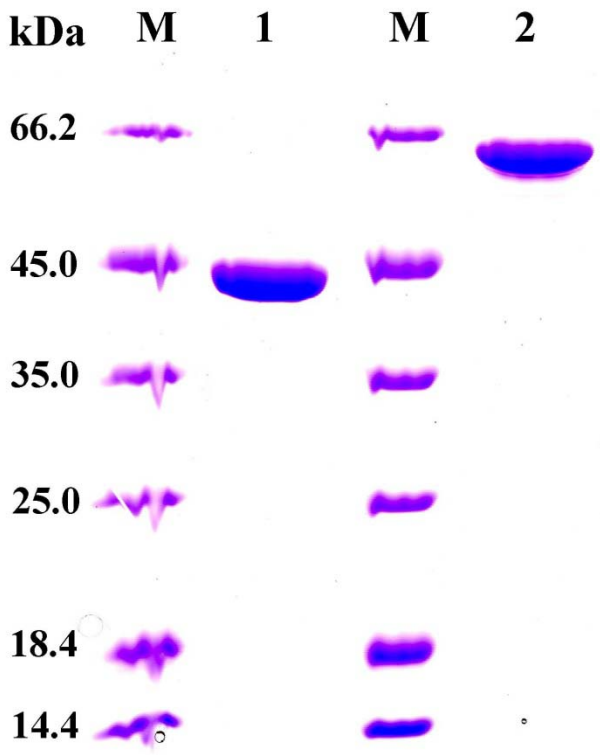

Figure S1 SDS–PAGE analysis of the purified Aldo and DHAD. M, marker proteins; line 1, purified Aldo; line 2, purified DHAD.

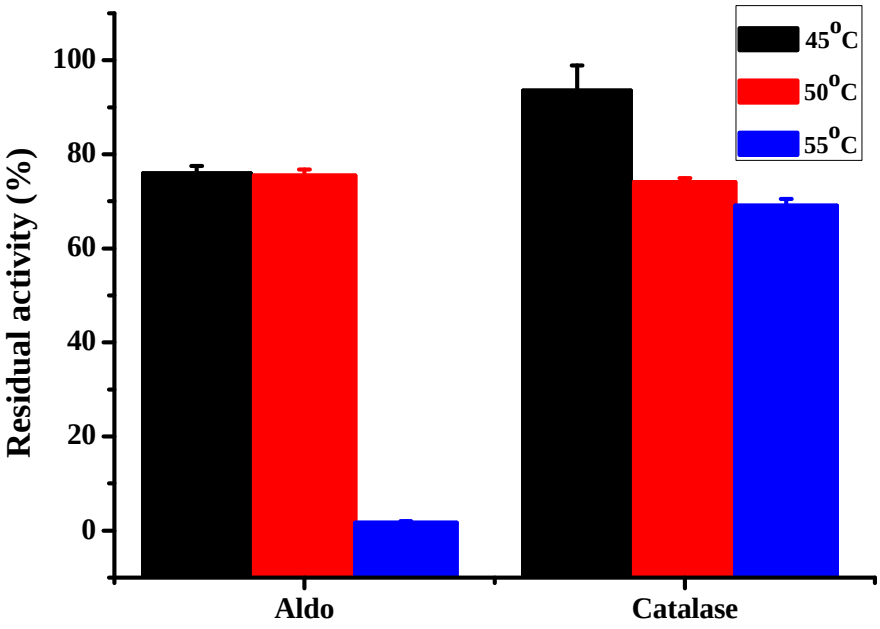

Figure S2 Thermostability of purified Aldo and catalase shown by residual activities. Activities of Aldo and catalase were measured after enzyme solutions had been incubated at various temperatures (45–55°C) for 12 h.

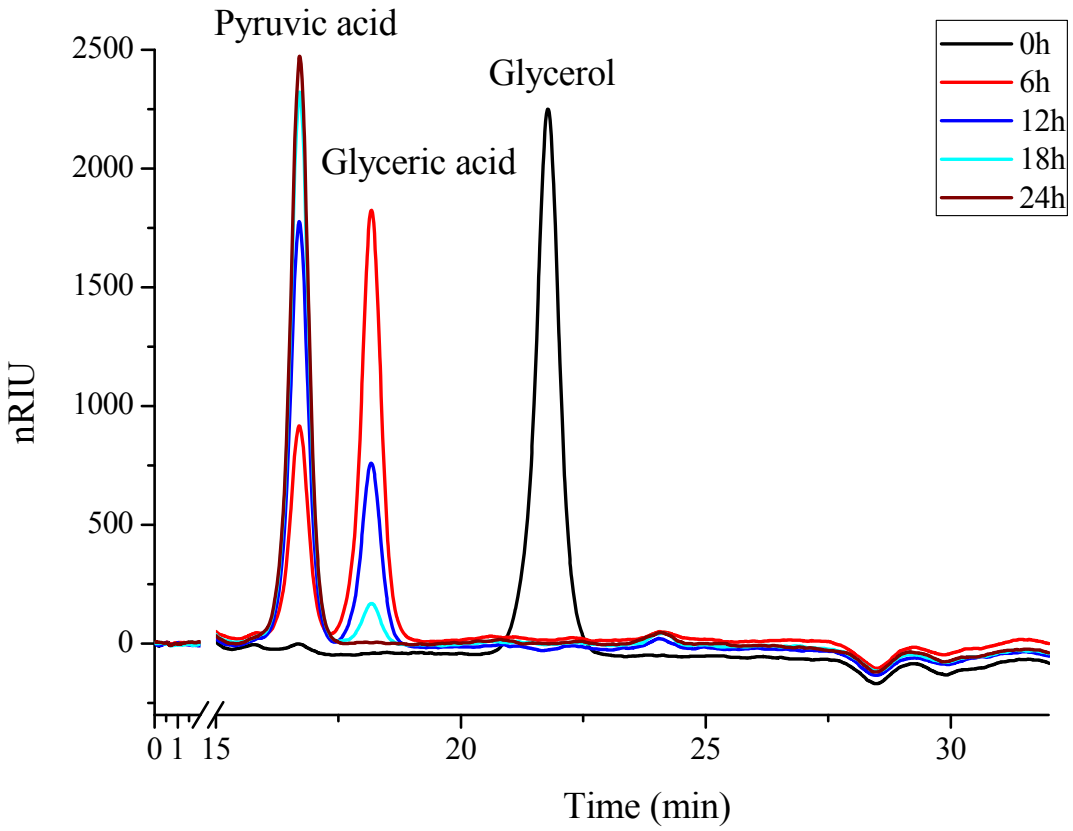

Figure S3 HPLC analysis of the reaction mixtures containing 100 mM HEPES buffer (pH 7.4), 10 mM glycerol, 0.3 U mL^{-1} Aldo, 0.1 U mL^{-1} DHAD, and 1000 U mL^{-1} catalase at 0 h, 6 h, 12 h, 18 h, and 24 h.

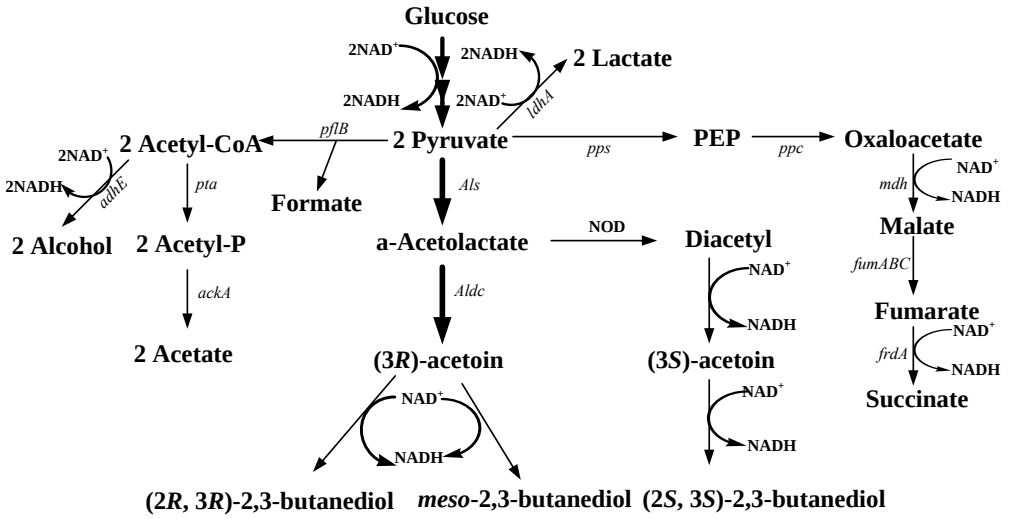

Figure S4 The mixed acid-2,3-butanediol pathway by microbes (modified based on reference 8 and 9). NOD: nonenzymatic oxidative decarboxylation; PEP: phosphoenolpyruvate.

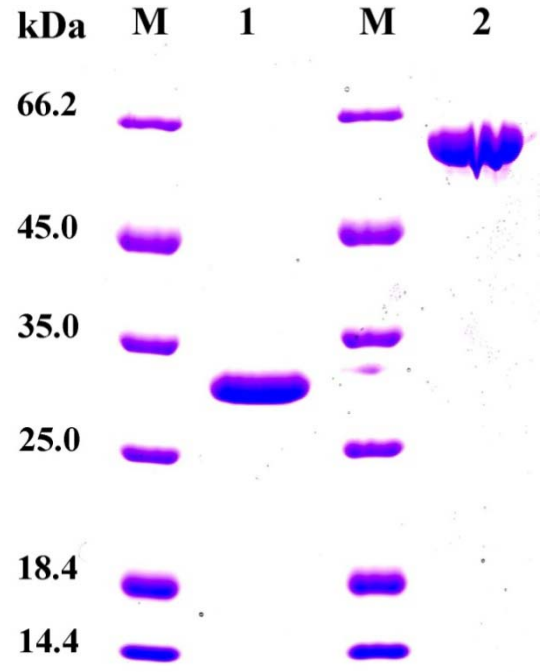

Figure S5 SDS–PAGE analysis of the purified ALS and ALDC. M, marker proteins; line 1, purified ALDC; line 2, purified ALS.

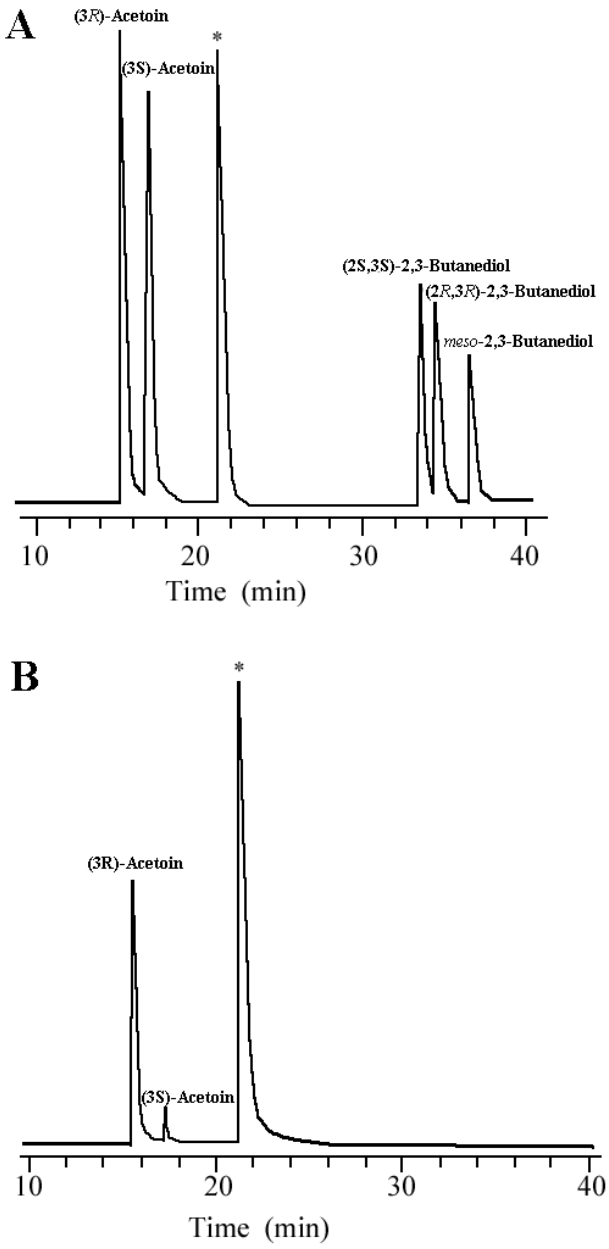

Figure S6 GC analysis of product of thecatalytic reaction (*Isoamyl alcohol was used as the internal standard). (A) GC analysis of (3*S*)-acetoin, (3*R*)-acetoin, *meso*-2,3-butanediol, (2*S*,3*S*)-2,3-butanediol and (2*R*,3*R*)-2,3-butanediol. (B) Product of the reaction mixture contained 100 mM HEPES buffer (pH 7.4), 10 mM glycerol, 0.2 mM thiamine diphosphate, 0.3 U mL^{-1} Aldo, 0.1 U mL^{-1} DHAD, and 1000 U mL^{-1} catalase, 200 U mL^{-1}ALS and 50 U mL^{-1} ALDC.

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