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Production of value-added chemicals from glycerol using in vitro enzymatic cascades

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The large surplus of glycerol derived from the expanding biofuel industry raises economic and environmental concerns regarding disposal. In vitro synthetic biology is emerging as a useful biomanufacturing platform while the conversion of glycerol is rarely investigated. Here we develop a thermostable in vitro synthetic biosystem consisting of three enzymatic cascades for the biotransformation of glycerol into valuable chemicals with different degrees of reduction. Condensation of glycerol, phenol, and ammonium into L-tyrosine is achieved using four enzymes without the assistance of NAD^+/NADH-related redox reactions. Production of chemicals with high degrees of reduction (e.g., optically pure L-lactate and D-lactate) is also verified through coupling with an NADH-regeneration system. The biotransformation of glycerol and ammonium into L-serine is achieved using four enzymes with self-sufficient NADH recycling.

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Amidst the increasing environmental concern and the declining availability of petroleum resources, the manufacturing of biofuels (including biodiesel and bioethanol) from sustainable biomass feedstocks has been increasing continuously throughout the world[1,2]. Glycerol is generated as an unavoidable byproduct during the manufacturing of both biodiesel and bioethanol[3]. During the transesterification process used in biodiesel production, 1 ton of glycerol is formed for every 9 tons of biodiesel produced[4]. The global annual production of glycerol is estimated to reach 4.2 million tons in 2020[5], while the demand for glycerol is predicted to be less than 3.5 million tons. In this context, the large surplus accumulation of glycerol has been a major burden to the biofuel industry and may hamper the widespread adoption of biofuels in the marketplace[6]. Thus, it is of great importance to explore novel approaches for the biotransformation of glycerol into value-added compounds, both for the development of the biodiesel industry and from the perspective of environmental protection.

In vitro synthetic biology, which involves the artificial design of metabolic pathways and assembly of catalytic elements, is emerging as a promising biomanufacturing platform[7–9]. Compared to the cell-based counterparts, in vitro synthetic systems are superior in many aspects, including: fast reaction rates resulting from the absence of transmembrane transport of substances[10], high product yields and easy purification of the target products resulting from the simplified metabolic processes involved[11], easy manipulation due to high tolerance to toxic chemicals and temperature fluctuations[12], shifting of unfavorable reaction equilibria[13], and the diversified design of metabolic pathways[14,15]. Previously, a variety of in vitro synthetic systems have been explored for the production of valuable chemicals based on renewable biomaterials such as glucose, xylose, sucrose, and glucan[11,12,14–19]. Among these systems, the production of biohydrogen through in vitro metabolic engineering has been studied extensively[11,14,18].

Previously, we designed an enzymatic cascade for the conversion of glycerol into pyruvate using three enzymes: alditol oxidase from *Streptomyces coelicolor* A3 (ALDO), dihydroxy acid dehydratase from *Sulfolobus solfataricus* (DHAD), and catalase from *Aspergillus niger*[20]. This process eliminates the complicated phosphorylation processes typically involved, but unfortunately lacks the ability to supply the reducing power required for the production of chemicals with higher degrees of reduction.

Here, to unlock the potential held in the accumulated surplus of glycerol, the biotransformation of glycerol into value-added chemicals with different reduction degrees is investigated in detail. Through the rational assembly of thermostable enzymes from various species, we construct a completely artificially designed in vitro biosystem for the production of valuable chemicals from glycerol.

Results

Production of L-tyrosine without demand for reducing power. Glycerol was first used to produce L-tyrosine, a chemical typically produced from pyruvate, without a demand for reducing power (Fig. 1). Since tyrosine phenol-lyase has previously been used to catalyze the condensation of phenol, pyruvate, and ammonium into L-tyrosine[21], the production of L-tyrosine from glycerol, phenol, and ammonium might be achieved through the coupling of the pyruvate production cascade with tyrosine phenol-lyase. Because higher reaction temperatures are beneficial for the mixing of substances, increasing the reaction rate, lowering the viscosity of the reaction mixture, and reducing the chances of bacterial contamination[12,20], thermostable enzymes were selected to verify the feasibility of the artificially designed enzymatic cascade. Tyrosine phenol-lyase from *Symbiobacterium thermophilum* (StTYRPL, Protein ID: WP_043714225.1) has previously been purified, characterized, and identified as being stable at high temperatures and over a broad range of pH values[21,22]. Thus, StTYRPL was overexpressed in recombinant form and purified to homogeneity (Supplementary Table 1 and Supplementary Fig. 1).

The production of L-tyrosine was carried out through mixing the four enzymes (ALDO, DHAD, catalase, and StTYRPL) with the substrates (glycerol, phenol, and ammonium). As shown in Fig. 2, 7.71 mM L-tyrosine, with an enantiomeric excess (*ee*) of greater than 99.9% (Supplementary Fig. 2), was produced from 10.0 mM glycerol in 77.1% yield. The amount of phenol consumed was consistent with the production of L-tyrosine. The production of L-tyrosine was nearly constant throughout the biotransformation process, implying that neither glycerol nor phenol significantly affected the activities or stabilities of the enzymes. No unwanted byproducts, such as acetate, ethanol, and succinate, which often accumulate during cellular metabolic processes, were detected. The L-tyrosine produced was also characterized by LC-MS (Supplementary Fig. 3).

Production of chemicals with high degrees of reduction. To produce chemicals with higher degrees of reduction from glycerol, the coupling of the NADH recycling system with the enzymatic reactions is technically feasible and easy to implement. Herein, the biotransformation of glycerol into L-lactate and D-lactate was carried out to verify the feasibility of such a system. Formate dehydrogenation was selected as the method of NADH regeneration since it does not introduce unwanted byproducts, thereby simplifying the purification of the target products. As shown in Fig. 1, the pyruvate produced from the oxidation of glycerol was reduced to L-lactate and D-lactate under catalysis by L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), respectively. As a result, one optical lactate molecule was produced from each glycerol molecule, at the cost of one NADH molecule donated from the dehydrogenation of formate.

Previously, D-LDH from *Thermodesulfatator indicus* (TiDLDH, Protein ID: WP_013906894) was used to produce D-lactate thermophilically; wherein high affinities and rapid conversion rates for both pyruvate and NADH were observed[23]. *Thermus thermophilus* HB8 is a hyperthermophilic bacterial strain, and many of its genes have been overexpressed and applied to thermophilic reactions[17]. Consequently, L-LDH from *T. thermophilus* HB8 (TtLLDH, Protein ID: YP_144379.1) was selected for L-lactate production. Formate dehydrogenase from *Ogataea parapolymorpha* DL-1 (OpFDH, Protein ID: EFW95288) has previously been characterized and was shown to possess promising thermostability, a wide pH optimum, and high organic tolerance[24]. All of these enzymes were overexpressed and purified (Supplementary Fig. 1). The kinetic parameters of these enzymes toward different substrates were determined (Table 1). OpFDH, TtLLDH, and TiDLDH were all thermostable and exhibited 79.0%, 81.1%, and 130.3% of their initial activities, respectively, after incubation at 50 °C for 24 h (Supplementary Fig. 4).

The production of optical L-lactate was carried out by mixing the five enzymes (ALDO, DHAD, catalase, TtLLDH, and OpFDH) with glycerol. NAD^+ was added at a concentration of 1 mM. As shown in Fig. 3a, 9.16 mM L-lactate with an *ee* of 100% (Supplementary Fig. 5) was generated from 10 mM glycerol over 24 h, in 91.6% yield. Formate was consumed alongside the production of L-lactate, and pyruvate was maintained at a concentration of below 0.5 mM throughout the catalytic process. The production of D-lactate was also achieved via replacing the TtLLDH with TiDLDH in the above procedure. As shown in Fig. 3b, 9.73 mM D-lactate with an *ee* of 100% (Supplementary

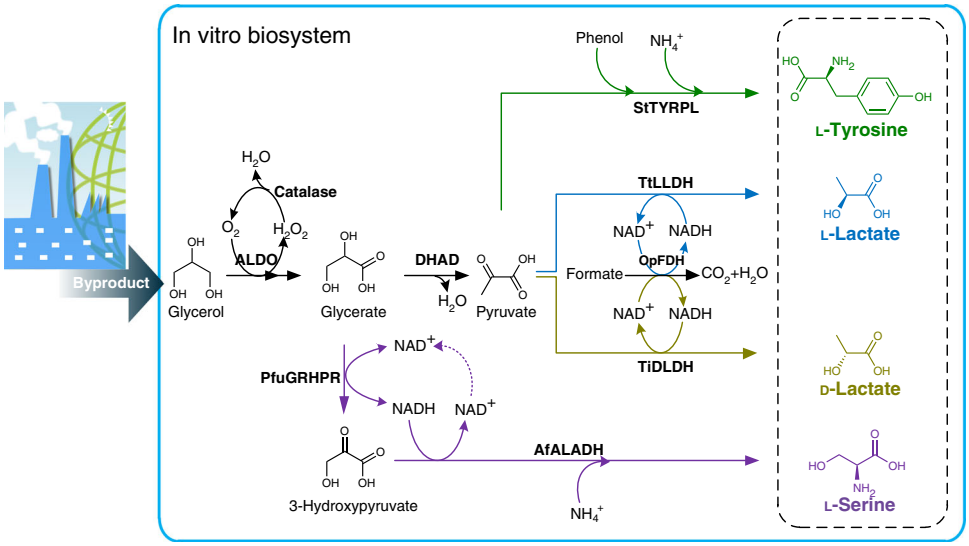

Fig. 1 Scheme of in vitro metabolic engineering of glycerol biotransformation. The colored lines and structures indicate the artificially designed pathways for production of L-tyrosine (green), L-lactate (blue), D-lactate (dark yellow), and L-serine (purple). ALDO alditol oxidase from *S. coelicolor* A3, DHAD dihydroxy acid dehydrogenase from *S. solfataricus*, OpFDH formate dehydrogenase from *O. parapolymorpha* DL-1, StTYRPL tyrosine phenol-lyase from *S. thermophilum*, TtLLDH L-lactate dehydrogenase from *T. thermophilus* HB8, TiDLDH D-lactate dehydrogenase from *T. indicus*, PfuGRHPR glyoxylate/hydroxypyruvate reductase from *P. furiosus*, AfALADH alanine dehydrogenase from *A. fulgidus*

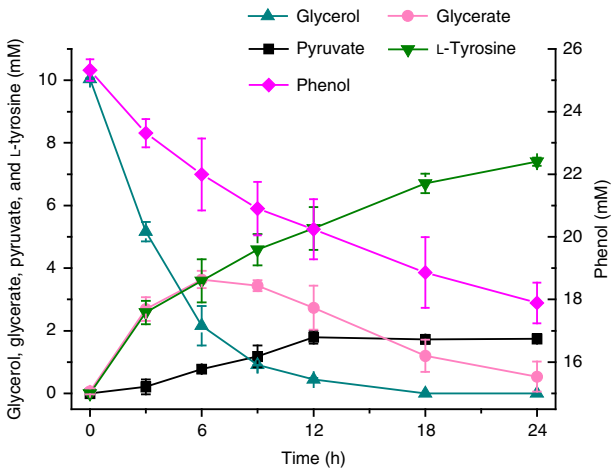

Fig. 2 Production of L-tyrosine from glycerol and phenol using enzymatic cascade. Reactions were carried out in 20 mL reaction mixture containing 0.3 U mL^{-1} ALDO (EC 1.1.3.41, with a specific activity of 0.26 U mg^{-1}), 0.1 U mL^{-1} DHAD (EC 4.2.1.39, with a specific activity of 0.011 U mg^{-1}), 1000 U mL^{-1} catalase (EC 1.11.1.6, with a specific activity of 131,600 U mg^{-1}), 12 U mL^{-1} StTYRPL (EC 4.1.99.2, with a specific activity of 1.96 U mg^{-1}), 0.5 mM pyridoxal 5′-phosphate (PLP), 10 mM glycerol, 25 mM phenol, 150 mM $(NH_4)_2SO_4$, and 100 mM HEPES-NaOH (pH 7.0) at 50 °C and 300 rpm. Error bars indicate standard errors of three parallel experiments

Fig. 5) was produced from 10 mM glycerol in 97.3% yield over 24 h. Compared to the yield of L-lactate, the higher yield of D-lactate may result from the low K_m of TiDLDH toward both pyruvate and NADH (0.05 and 0.03 mM, respectively)[23]. No unwanted byproducts were detected during the entire biotransformation process for either L-lactate or D-lactate production.

Production of L-serine with self-sufficient NADH recycling. To further extend the possible utility of glycerol, we designed a completely artificial pathway for the conversion of glycerol and ammonium into L-serine, with self-sufficient NADH recycling. The synthetic pathway was composed of four enzymes and did not require the addition of costly ATP or ADP (Fig. 1). The glycerate generated from the oxidation of glycerol was converted into 3-hydroxypyruvate under the catalysis of NAD^+-dependent glyoxylate/hydroxypyruvate reductase. However, the standard Gibbs free energy (Δ_rG^θ) of this reaction was estimated to be 20.26 kJ mol^{-1} (the calculation procedure for Δ_rG^θ is shown in Supplementary Note 1). In this context, the substrate promiscuity of alanine dehydrogenase was exploited to catalyze the reductive amination of 3-hydroxypyruvate into L-serine ($\Delta_rG^\theta = -69.64$ kJ mol^{-1}). Consequently, Δ_rG^θ for the production of L-serine from glycerate and ammonium was estimated to be −49.38 kJ mol^{-1}, which ensured that these reactions were thermodynamically feasible. Theoretically, one molecule of L-serine could be produced from one molecule of glycerol and one molecule of ammonium. The glyoxylate/hydroxypyruvate reductase from the hyperthermophilic archaea *Pyrococcus furiosus* (PfuGRHPR, Protein ID: WP_011011434.1) has been reported to catalyze the reduction of 3-hydroxypyruvate with high catalytic efficiency (k_{cat}/K_m)[25]. The L-alanine dehydrogenase from another hyperthermophilic archaea *Archaeoglobus fulgidus* (AfALADH, Protein ID: WP_010879161.1) has been shown to be able to catalyze the reductive amination of 3-hydroxypyruvate into L-serine[26]. Based on these functionalities, PfuGRHPR and AfALADH were overexpressed and characterized (Supplementary Fig. 1).

Since the activity of PfuGRHPR toward the oxidation of glycerate was difficult to assay, its activity toward the reduction of hydroxypyruvate was determined using NADH as the cofactor. As shown in Supplementary Fig. 6, the activity of PfuGRHPR toward hydroxypyruvate reduction decreased sharply in the pH range of 7.0–9.0, while the reductive amination activity of AfALADH increased in the same range. For the conversion of glycerate into L-serine, the reductive activity of PfuGRHPR should be restricted, while the reductive amination activity of AfALADH should be promoted. In this regard, a pH of 9.0 was chosen for the production of L-serine. The kinetic parameters of the two enzymes toward different substrates were also determined (Table 1). PfuGHPR and AfALADH were both thermostable,

Table 1 Kinetic parameters of enzymes used in this study toward different substrates. Results are means ± SD of three independent experiments

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}·s^{-1}$)
OpFDH	NAD^+	168.19 ± 7.22	4.86 ± 0.47	28.89 ± 2.79
	Formate	4446.87 ± 131.94	4.24 ± 0.67	0.95 ± 0.15
TtLLDH	NADH	1317.31 ± 15.69	55.14 ± 4.60	41.86 ± 3.49
	Pyruvate	13,668.21 ± 432.45	14.01 ± 4.50	1.03 ± 0.33
	L-Lactate	ND[a]	ND	ND
PfuGRHPR	NADH	128.85 ± 47.83	2.56 ± 0.01	19.84 ± 0.08
	3-Hydroxypyruvate	UD[b]	UD	UD
AfALADH	NADH	1.30 ± 0.06	1.94 ± 0.01	1496.99 ± 0.09
	3-Hydroxypyruvate	1540.45 ± 169.05	2.03 ± 0.13	1.32 ± 0.09

[a]ND means not detected
[b]UD means unable to be detected since it did not fit the Michaelis-Menten equation

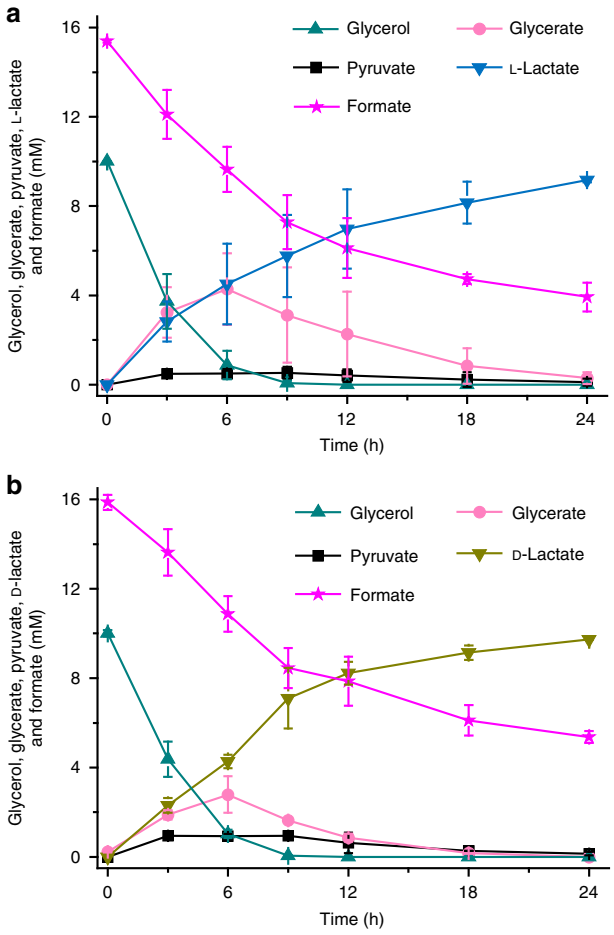

Fig. 3 Production of optically pure L-lactate and D-lactate from glycerol using enzymatic cascades. **a** Production of L-lactate; **b** Production of D-lactate. Reactions were carried out in 20 mL reaction mixture containing 0.3 U mL^{-1} ALDO, 0.1 U mL^{-1} DHAD, 1000 U mL^{-1} catalase, 3 U mL^{-1} TtLLDH (EC 1.1.1.27, with a specific activity of 0.54 U mg^{-1}) or TiDLDH (EC 1.1.1.28, with a specific activity of 30.2 U mg^{-1}), 6 U mL^{-1} OpFDH (EC 1.17.1.9, with a specific activity of 6.11 U mg^{-1}), 10 mM glycerol, 15 mM sodium formate, 1 mM NAD^+, and 100 mM HEPES-NaOH (pH 7.0) at 50 °C and 300 rpm. Error bars indicate standard errors of three parallel experiments

exhibiting 110.3% and 64.9% of their initial activities, respectively, after incubation for 24 h at 50 °C and pH 9.0 (Supplementary Fig. 7).

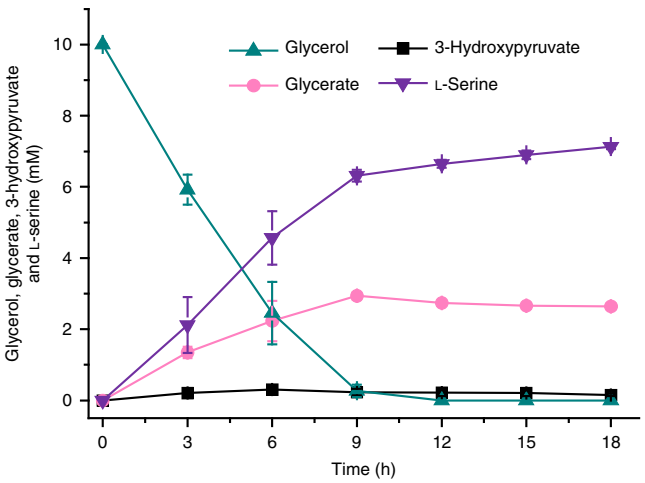

Fig. 4 Production of L-serine from glycerol using enzymatic cascade. Reactions were performed in 20 mL reaction mixture containing 0.3 U mL^{-1} ALDO, 1000 U mL^{-1} catalase, 7 U mL^{-1} PfuGRHPR (EC 1.1.1.29, with a specific activity of 2.35 U mg^{-1}), 30 U mL^{-1} AfALADH (EC 1.4.1.1, with a specific activity of 3.12 U mg^{-1}), 10 mM glycerol, 1 mM NAD^+, 150 mM $(NH_4)_2SO_4$, and 100 mM HEPES-NaOH (pH 9.0) at 50 °C and 300 rpm. Error bars indicate standard errors of three parallel experiments

As shown in Fig. 4, 7.13 mM L-serine with an *ee* of over 99.9% (Supplementary Fig. 8) was produced from 10.0 mM glycerol over 18 h, in 71.3% yield. The concentration of 3-hydroxypyruvate was kept low (below 0.3 mM) during the catalytic process. Glycerate, at a concentration of 2.36 mM, was left as the major byproduct, and no other unwanted byproducts were detected. The fermentative production of L-serine was restricted by the feedback-based inhibition of D-3-phosphoglycerate dehydrogenase by L-serine[27,28]. The residual glycerate might also be due to similar feedback-based inhibition of the enzymes of the in vitro biosystem by L-serine.

Discussion

Amidst growing concerns about global energy and anthropogenic climate change, biofuels are believed to be promising alternatives to replace traditional fossil fuels[29]. Accompanying the rapid expansion of biofuel industry is surplus of glycerol, the main byproduct of biofuel production[30]. The low price and the large quantity availability of glycerol have made it an ideal feedstock for the production of various chemicals[30–32]. Herein, we have

constructed an in vitro biosystem for the biotransformation of glycerol into value-added chemicals with different degrees of reduction.

Glycerol can be converted into pyruvate by an artificial enzymatic reaction cascade composed of ALDO, DHAD, and catalase. Through reactions not involving the participation of NAD^+/NADH, pyruvate can be directly used as the substrate for the production of various chemicals such as N-acetylneuraminate, acetoin, and L-tyrosine, the latter of which is illustrated in this study (Figs. 1 and 2). The oxidation of glycerol by O_2 does not provide the reducing power required for the production of chemicals with higher degrees of reduction such as lactate, alanine, 2,3-butanediol and ethanol. Thus, coupling the enzymatic pathway with an NADH-regeneration system using formate and formate dehydrogenase was thought to be a potential solution to this problem. The production of both L-lactate and D-lactate in high yields and optical purities verified this assumption (Figs. 1 and 3). During the production of pyruvate from glycerol, glycerate would be produced as an intermediate product. Besides being dehydrated to produce pyruvate, glycerate can also be dehydrogenated to produce 3-hydroxypyruvate and NADH, which might be useful for chemical production processes involving self-sufficient NADH recycling. Herein, the manufacturing of L-serine from glycerol and ammonium is achieved by applying this concept (Figs. 1 and 4).

Both L-tyrosine and L-serine serve as valuable precursors with multiple applications in the food, chemical, pharmaceutical, and cosmetic industries[33,34]. However, the fermentative syntheses of L-tyrosine and L-serine are restricted by the multistep reactions required and complex regulatory processes. For example, the cellular synthesis of L-serine from glycerol requires at least eight enzymes. Complicated phosphorylation and inhibitory feedback mechanisms are also involved in the metabolic processes (Supplementary Fig. 9). In this study, two artificially designed enzymatic cascades were constructed to manufacture L-tyrosine and L-serine from glycerol, using the minimum number of enzymes. L-Tyrosine, with an *ee* greater than 99.9%, was produced in 77.1% yield. Further, L-serine, with an *ee* of greater than 99.9%, was produced from glycerol in 71.3% yield, which is higher than any microbial fermentation processes reported to date[35].

Optically pure lactate is a platform chemical that can be utilized in many industrial applications[36,37]. Nowadays, lactate isomers can be produced with high optical purity through microbial processes. However, a novel technology that can produce lactate from inexpensive raw materials and in high optical purity is still desirable for supporting the potential applications of optically pure lactate. In this study, optically pure L-lactate and D-lactate (*ee* 100%) were produced from glycerol in high yields. NADH regeneration was achieved through coupling the system with a thermostable formate dehydrogenase, which avoided the production of unwanted byproducts.

The yields and production rates of the systems developed in this study still need to be improved before their industrial applications. When 50 g L^{-1} glycerol was added in the reaction system for L-lactate production, only 34.4 mM L-lactate was produced in 72 h with a productivity of 0.48 mM h^{-1} (Supplementary Fig. 10). The low activities of some key enzymes such as ALDO (0.26 U mg^{-1}) and DHAD (0.011 U mg^{-1}) may be the limiting factors requiring future studies. New biocatalysts with high activities, obtained through systematic screening or directed evolution, will be necessary to expand the potential applications of the in vitro synthetic systems. Considering the instability of NAD^+ at high temperatures, enzymes that are capable of using thermostable and cheap NAD^+ analogs might promote the application of the in vitro biosystem. Systematic optimization of the reaction conditions may also improve the performance of these systems[14].

In summary, the large surplus of glycerol derived from the dramatic growth of the biofuel industry has caused economic and environmental concerns regarding its disposal. Using selected thermostable enzymes, we designed a completely artificial in vitro biosystem involving different enzymatic cascades to biotransform glycerol into value-added chemicals with different degrees of reduction. Manufacturing of L-tyrosine was achieved through the condensation of glycerol, ammonium, and phenol without the assistance of NAD^+/NADH-related redox reactions. Optically pure L-lactate and D-lactate were produced through coupling with an NADH regeneration system. Production of L-serine from glycerol and ammonium was achieved using a four-enzyme cascade. The in vitro enzymatic system may be a versatile and useful platform for the production of value-added chemicals from glycerol.

Methods

Reagents. Authentic D,L-lactate, D-lactate, L-lactate, D-tyrosine, L-tyrosine, D-serine, L-serine, 3-hydroxypyruvate, and D,L-glycerate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction endonuclease and T4 DNA ligase were purchased from Thermo Scientific (Lithuania). FastPfu DNA polymerase was purchased from TransGen Biotech Ltd. (Beijing, China). The oligonucleotides were synthesized by Sangon Biotech Ltd. (Shanghai, China). The genomic DNA of *T. thermophilus* HB8 (DSM 579) was purchased from Leibniz-Institut DSMZ (Braunschweig, Germany). All the other chemicals were of analytical pure grade and commercially available.

Construction of recombinant strains. The strains, plasmids, and primers used in this study are listed in the Supplementary Table 1. *Escherichia coli* DH5α was used for plasmid construction and *E. coli* BL21(DE3) was used for overexpression of these recombinant proteins. Codon optimized encoding genes of OpFDH, StTYRPL, PfuGRHPR, and AfALADH (the optimized nucleic acid sequences for expression in *E. coli* can be found in the Supplementary Data 1) were synthesized by General Biosystems, Inc. (Chuzhou, China) and inserted into plasmid pETDuet-1. The resulting recombinant plasmids were then transferred into *E. coli* BL21 (DE3) to construct *E. coli* BL21(DE3)/pETDuet-OpFDH, *E. coli* BL21(DE3)/pETDuet-StTYRPL, *E. coli* BL21(DE3)/pETDuet-PfuGRHPR, and *E. coli* BL21 (DE3)/pETDuet-AfALADH (Supplementary Table 1) for enzyme overexpression. Nucleotide sequence encoding gene of TtLLDH was amplified from the genomic DNA of *T. thermophilus* HB8 with the primers TtLLDH.f/TtLLDH.r (Supplementary Table 1) and then ligated into restriction sites of *BamH*I/*Hind*III of plasmid pETDuet-1. The recombinant plasmid pETDuet-TtLLDH was transferred into *E. coli* BL21(DE3) to construct *E. coli* BL21(DE3)/pETDuet-TtLLDH for TtLLDH overexpression.

All the *E. coli* strains were cultivated in LB medium at 37 °C and 180 rpm. If necessary, ampicillin was added into the medium at a final concentration of 100 μg mL^{-1}.

Preparation of enzymes. Catalase from *A. niger* was purchased from Sigma-Aldrich (St. Louis, MO, USA). To prepare ALDO, DHAD, TiDLDH, OpFDH, TtLLDH, StTYRPL, PfuGRHPR, and AfALADH, the recombinant strains were cultivated at 37 °C to early logarithmic phase (OD_{600nm} = 0.6–0.8). Then, IPTG at a final concentration of 1 mM was added into the culture medium. The culture was cultivated for another 12 h at 20 °C for protein overexpression. Cells were harvested and washed twice with 67.7 mM phosphate solution buffer (PBS, pH 7.40).

The recombinant strains were resuspended in binding buffer (20 mM phosphate buffer, 500 mM NaCl, and 20 mM imidazole, pH 7.40) supplemented with 1.0 mM phenylmethylsulfonyl fluoride (PMSF) to prevent proteolysis. Cells were lysed using a continuous high pressure cell disrupter working at 4 °C and 1200 bar. The cell lysate was incubated at 50 °C for 45 min before being centrifuged at 12,000 rpm for 1 h to remove the protein precipitation, cell debris, and intact cells. The supernatant was filtered using a 0.22 μm poly(ether sulfone) (PES) filter, and then loaded onto a 5 mL HisTrap HP column (GE Healthcare, Sweden). Target proteins were eluted with a gradient ratio of elution buffer (20 mM phosphate buffer, 500 mM NaCl, and 500 mM imidazole, pH 7.40), concentrated by ultrafiltration, desalted using a 5 mL HisTrap desalting column (GE Healthcare, Sweden), frozen in liquid nitrogen, and preserved at −80 °C until use. The purified proteins were subjected to SDS-PAGE, and the results are shown in Supplementary Fig. 1.

Determination of enzymatic characteristics. The activities of OpFDH, TtLLDH, TiDLDH, PfuGRHPR, and AfALADH were analyzed photometrically by monitoring the absorption of NADH at 340 nm using a UV–visible spectrophotometer

(Ultrospec 2100 pro; Amersham Biosciences) with a thermostat water bath. All the reactions were buffered with HEPES-NaOH (100 mM, pH 7.0) and carried out in a 1 mL quartz cuvette at 50 °C. To determine the kinetic parameters of OpFDH, either 0–40 mM sodium formate (with 1 mM NAD^+) or 0–5 mM NAD^+ (with 20 mM sodium fromate) was added. Reactions were started with the addition of appropriate amount of OpFDH. Michaelis-Menten equation was used for calculation of K_m and k_{cat}. Similarly, the kinetic parameters of TtLLDH were estimated with the addition of either 0–100 mM sodium pyruvate (with 0.2 mM NADH) or 0–1 mM NADH (with 20 mM sodium pyruvate). To determine the enzymatic activity of PfuGRHPR and AfALADH, 3-hydroxypyruvate was used as the substrate. The kinetic parameters of PfuGRHPR toward NADH were determined through the addition of 0–0.2 mM NADH (with 5 mM 3-hydroxypyruvate). The kinetic parameters of AfALADH toward NADH and 3-hydroxypyruvate were estimated through the addition of either 0–0.2 mM NADH (with 10 mM 3-hydroxypyruvate) or 0–10 mM 3-hydroxypyruvate (with 0.2 mM NADH). $(NH_4)_2SO_4$ at a final concentration of 150 mM was added into the buffer, and the pH was set at 9.0. Three independent experiments were performed for determining the kinetic parameters.

The thermostabilities of OpFDH, TtLLDH, TiDLDH, PfuGRHPR, and AfALADH were characterized through monitoring the remaining enzyme activities after incubation at 50 °C for indicated time intervals.

Cascade reactions. In a previous study, the biotransformation of glycerol into pyruvate was achieved using 0.3 U mL^{-1} ALDO, 0.1 U mL^{-1} DHAD, 1000 U mL^{-1} catalase from *A. niger*[20]. The content and ratio of ALDO and DHAD was verified to efficiently convert glycerol into pyruvate. Excess catalase was added to eliminate the H_2O_2 generated from glycerol oxidation. StTYRPL, OpFDH, TtLLDH, TiDLDH, PfuGRHPR, and AfALADH have higher specific activities than the restricting enzymes ALDO and DHAD. In this study, these enzymes were also added with excess concentrations like the catalase. The L-tyrosine production was carried out in 20 mL reaction mixture containing 0.3 U mL^{-1} ALDO, 0.1 U mL^{-1} DHAD, 1000 U mL^{-1} catalase from *A. niger*, 12 U mL^{-1} StTYRPL, 0.5 mM pyridoxal 5'-phosphate (PLP), 10 mM glycerol, 25 mM phenol, 150 mM $(NH_4)_2SO_4$, and 100 mM HEPES-NaOH (pH 7.0).

To produce optically pure L-lactate and D-lactate from glycerol and formate, reactions were carried out in 20 mL reaction mixture containing 0.3 U mL^{-1} ALDO, 0.1 U mL^{-1} DHAD, 1000 U mL^{-1} catalase from *A. niger*, 3 U mL^{-1} TtLLDH or TiDLDH, 6 U mL^{-1} OpFDH, 10 mM glycerol, 15 mM sodium formate, 1 mM NAD^+, and 100 mM HEPES-NaOH (pH 7.0).

Biotransformation of glycerol and ammonia into L-serine was performed in 20 mL reaction mixture containing 0.3 U mL^{-1} ALDO, 1000 U mL^{-1} catalase, 7 U mL^{-1} PfuGRHPR, 30 U mL^{-1} AfALADH, 10 mM glycerol, 1 mM NAD^+, 150 mM $(NH_4)_2SO_4$, and 100 mM HEPES-NaOH (pH 9.0).

All these reactions were performed in 50 mL conical flasks which were incubated in a thermostatic waterbath shaking at 150 rpm and 50 °C. Three parallel experiments were carried out for each enzymatic cascade reaction. Samples (0.2 mL) were withdrawn at the indicated times before being heat treated for analysis on HPLC. For L-tyrosine production, samples were mixed with 1 N H_2SO_4 (1:1, v/v) to stop the reaction and dissolve L-tyrosine.

Analytical methods. The protein concentration was determined using the Bradford protein assay (Bio-Rad). The concentrations of glycerol, glyceraldehyde, glycerate, pyruvate, lactate, formate, and 3-hydroxypyruvate were analyzed using an HPLC system (Agilent 1100 series, Hewlett-Packard, USA) equipped with an anion exchange column (Aminex HPX-87H, 300 × 7.8 mm; Bio-Rad, USA) and a refractive index detector, as described by Li et al.[38]. The analysis was carried out at 55 °C using 10 mM H_2SO_4 as the mobile phase. The flow rate was 0.4 mL min^{-1}. Stereoselective assays of lactate and tyrosine were performed using an HPLC system equipped with a chiral column (CRS10W, 4.6 mm × 50 mm) and a UV–Vis detector working at 254 nm. The elution was performed at 25 °C using 2 mM $CuSO_4$ as the mobile phase. The flow rate was 0.5 mL min^{-1}. Similarly, stereoselective assays of serine were carried out using 0.1 mM $CuSO_4$ as the mobile phase.

The concentration of tyrosine and phenol was analyzed using an HPLC system equipped with a UV–Vis detector working at 280 nm. The separation was achieved with a reverse phase C18 column (Agilent Eclipse XDB-C18, 4.6 mm × 150 mm, 5 μm) at 40 °C. The elution was carried out using a co-solvent consisting of 100 mM KH_2PO_4 (pH 2.10) (A) and acetonitrile (B) at a volume ratio of 3:1 and a flow rate of 1 mL min^{-1}.

Serine concentration was quantified using an HPLC system equipped with a UV–Vis detector set at 360 nm. To avoid the influence of ammonium ion on the derivatization of serine, the samples was diluted to suitable multiples. One milliliter of diluted sample was mixed with 0.5 mL $NaHCO_3$ (0.5 M) and 0.5 mL 2,4-dinitrofluorobenzene (DNFB)-acetonitrile (1 mL DNFB in 100 mL acetonitrile), incubated at 60 °C for 1 h, cooled to room temperature, mixed with 8 mL KH_2PO_4 (20 mM, pH 7.0), and then centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through a 0.22 μm membrane before being injected into the HPLC system. The separation was achieved using a C18 column at 40 °C with 50 mM sodium acetate (A) and water/acetonitrile (volume ratio of 1:1) (B) as the mobile phase (70% A) at a flow rate of 1 mL min^{-1}.

The combination of high-performance liquid chromatography and mass spectrometry was performed to identify the end-product of L-tyrosine production cascade.

Data availability

The authors declare that all the other data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding authors upon request.

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Author contributions

C.G. and Z.L. designed the project. C.M., C.G. and P.X. supervised the project. Z.L. performed the experiments and analyzed the experimental data. J.Y. and J.S. helped the purification of the enzymes. Z.L., C.G., C.M. and P.X. wrote the paper.

Additional information

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